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*School of Pharmacy/ Department of Anatomy & Neuroscience*

# Ghrelin system signalling in appetite and reward: *in vitro* and *in vivo* perspectives

*Thesis presented by*

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*under the supervision of*

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*for the degree of*

**Doctor of Philosophy**

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# Table of Contents

## List of Appendices



## Glossary of Terms

| | |
|---|---|
| GHSR-1a | Growth Hormone Secretagogue Receptor-Type 1a |
| BBB | Blood Brain Barrier |
| GPCR | G-Protein Coupled Receptor |
| CNS | Central Nervous System |
| CSF | Cerebrospinal Fluid |
| NTS | Nucleus Tractus Solitarius |
| CCK | Cholecystokinin |
| GH | Growth Hormone |
| GHRP | Growth Hormone Releasing Peptide |
| DIO | Diet Induced Obesity |
| VTA | Ventral Tegmental Area |
| NAcc | Nucleus Accumbens |
| LH | Lateral Hypothalamus |
| PBN | Parabrachial Nucleus |
| GHSR-1a | Growth Hormone Secretagogue Receptor-Type 1a |
| CasHyd | Casein Hydrolysate (MF1145) |

FHI-2571 Whey Hydrolysate (UL-2-141)

RKT Rikkunshito

HEK Human Embryonic Kidney

RFU Relative Fluorescence Unit

EGFP Enhanced Green Fluorescent Protein

FBS Fetal Bovine Serum

CFI Cumulative Food Intake

SGF Simulated Gastric Fluid

# Declaration

I hereby declare that the submitted thesis consists of my own work and has not been submitted for another degree, at either University College Cork or elsewhere. I am familiar with regulations of plagiarism of University College Cork, and in my knowledge state that no part of this report is plagiarized.

Signed:

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# Author Contributions

All of the work conducted in this thesis was performed independently by myself, Ken Howick, with the following exceptions; all dairy-derived hydrolysates used in this thesis were provided by Moorepark Food Research Centre, Teagasc and the University of Limerick, in collaboration with Food for Health Ireland. Fractionation, compositional analysis and reversed-phase high performance liquid chromatography (RP-HPLC) profiles of hydrolysates were performed by colleagues in Food for Health Ireland, who are all co-authored in the relevant publications (Chapters 2 & 3). The *in vitro* calcium mobilization assays (Chapter 2 & 3) were carried out by Dalia Kandil, Mert Calis and Barbara Chruścicka. *In vitro* assays calcium mobilization, β-arrestin, internalization and IP-one (Chapter 4) were carried out by Lucas Van Leeuven, Shauna Wallace and Valerie Ramirez. The BARDS technique described in Chapter 3 was carried out by Ryan Alam. Scanning Electron Microscopy was performed with the help of Suzanne Crotty.

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The completion of a thesis is not possible without the help and dedication of numerous individuals. I was lucky to benefit from the wealth of experience of a wide circle of brilliant scientists within the Department of Anatomy & Neuroscience, of which there are too many to name individually. To the Schellekens lab group – you were a great team to be a part of and I enjoyed working with you all. In particular, I thank Dr. Thorsten Becker for his constant willingness to help and tutor me throughout his time in Cork. I was also fortunate to be affiliated with the School of Pharmacy and would like to thank all of its postgrads, past and present, again too many to mention. Additionally, I wish to acknowledge the UCC staff - sincere thanks to Tara Foley, Pat Fitzgerald, Gerry Moloney, Ger Clarke, Ken Devine, Tom O'Mahony and Áine Healy. I also express my gratitude to Colette Manley and all of the BSU staff, whose work is often underappreciated. The travails of a PhD are undoubtedly made easier by those who can empathize with your frustration. Thanks to my friends Erin, Ciarán, Seamus, Shauna, Cara, Ruth and the rest of the Bagel Box gang for all the morning coffee chats. Best of luck to you all in your future endeavours.

Life is not all about work; throughout my PhD I am lucky to have been surrounded by a loyal family and friends who maintained balance and perspective during the 4 years. I owe my closest friend Barry a debt of gratitude for listening to my endless complaints, as well as my sister, Katie, and brother, Kieran, for always being there. To my fiancée Niamh, your infinite patience, kindness and encouragement were a constant comfort - here's to the future. Finally, my Mam and Dad have worked tirelessly to afford me the opportunity to achieve my goals. Words can't describe how much I appreciate them.

# Publications and presentations

*Peer-reviewed publications*

**Howick, K**.; Griffin, B.T.; Cryan, J.F.; Schellekens, H. From belly to brain: Targeting the ghrelin receptor in appetite and food intake regulation. *Published,* Int J Mol Sci 2017.

**Howick, K**.; Alam, R.; Chruscicka, B.; Kandil, D.; Fitzpatrick, D.; Ryan, A.M.; Cryan, J.F.; Schellekens, H.; Griffin, B.T. Sustained-release multiparticulates for oral delivery of a novel peptidic ghrelin agonist: Formulation design and *in vitro* characterization. *Published,* Int J Pharm 2018, 536, 63-72.

**Howick, K**.; Wallace-Fitzsimons, S.; Kandil, D.; Chruścicka, B.; Calis, M.; Murphy, E.; Murray, B.; Fernandez, A.; Barry, K.; Kelly, P., et al. A dairy-derived ghrelinergic hydrolysate modulates food intake *in vivo*. *Published,* Int J Mol Sci, 2018.

**Howick, K**., Chruscicka, B, Ramirez, V, van Leeuven, L, Pietra, C. Cryan, J.F, Griffin, B.T, Schellekens, H. Behavioural characterization of novel ghrelin ligands, anamorelin and HM01: Appetite and reward-motivated effects in rodents. Journal of pharmacology and Experimental Therapeutics, *in preparation.* Jnl Pharm Exp Ther, 2018.

**Howick, K**., Clarke, G, Fitzgerald, P, V, Pietra, C. Cryan, J.F, Griffin, B.T, Schellekens, H. Effects of novel ghrelin ligands, anamorelin and HM01 on the reward circuitry: A microdialysis study in rodents, *in preparation,* Neuroscience Letters

*Poster presentations:*

**Howick, K**.; Chruścicka, B.; Ramirez, V; van Leeuven, L; Pietra,C; Cryan, J.F.; H. Griffin, B.T; Schellekens, H. Behavioural characterization of novel ghrelin ligands, Anamorelin and HM01: Appetite and reward-motivated effects in rodents. 11th FENS Forum of Neuroscience, Berlin, Germany. 20th - 24th May 2017.

**Howick, K**.; Schellekens, H; Alam, R; Kandil, D; Cryan, J.F.; H. Griffin, B.T. A platform for oral delivery of a novel ghrelin agonist peptide: formulation design and *in vitro* characterization. 6th Pharmaceutical Sciences World Congress, Stockholm, Sweden. 7th – 11th July 2018.

**Howick, K**.; Schellekens, H; Cryan, J.F.; H. Griffin, B.T. A platform for oral delivery of a novel ghrelin agonist peptide: formulation design and *in vitro* characterization. All-Ireland Schools of Pharmacy Conference, University College Cork. 24th - 25th April 2017.

# Thesis Summary

The regulation of food intake is one of the most intricate internal balances in mammalian behaviour. Dysregulation of the central mechanisms underlying appetite control and metabolism result in both disorders of under- and over-eating. Disorders of appetite result in significant morbidity and mortality, and represent a major unmet clinical need. The endogenous hormone ghrelin and its receptor, the growth hormone secretagogue receptor (GHSR-1a), have long been known as pharmacological targets for appetite-related and metabolic disorders. Nutraceutical and bioactive peptides offer the opportunity to prevent onset and escalation of lifestyle-associated diseases of appetite and metabolism. However, there is a dearth of clinical evidence to justify the development of many bioactives as nutraceuticals. The potential applicability of dairy-derived bioactives in appetite-related disorders is now becoming increasingly apparent. We investigate whether a dairy-derived hydrolysate can increase GHSR-1a signalling *in vitro,* and whether this can be translated to evidence of effect *in vivo* in a pre-clinical model (Chapter 2). Subsequently, by leveraging advanced pharmaceutical technology, we develop a gastro-protective and sustained delivery system with a high payload capacity (Chapter 3). Furthermore, ligand-dependent biased signalling, and ligand biodistribution may have important roles to play in increasing efficacy of ghrelin ligands *in vivo.* Therefore, we investigate whether two synthetic ghrelin ligands, anamorelin and HM01, exert differential effects on the GHSR-1a *in vitro* (Chapter 4). The divergent effects of these two ligands on appetite and reward-motivated behaviours, as well as effects on central neuronal activation and reward system dopamine (DA) levels will also be investigated with a view to informing strategies to optimize future ghrelin therapies (Chapter 4 and 5).

Chapter 2 and 3 provide an effective platform for gastro-protected delivery of bioactive peptides to enable further proof-of-concept studies across the appetite modulation field. Evidence of an orexigenic effect of the bioactive is seen *in vivo* in a rodent model. The oral delivery system developed served as a clinical formulation platform for proof-of-concept studies in humans to be conducted within the wider Food for Health Ireland research consortium. Chapters 4 and 5 show the importance of biased signalling and biodistribution of ghrelin ligands. Greater maximal food

intake is reported by the brain penetrant HM01 vs. the peripherally limited anamorelin. Divergent neuronal activation of the two ligands is also shown in reward processing areas using c-fos immunostaining. Targeting specific downstream signalling pathways will enable the provision of more efficacious appetite modulation therapies, while centrally penetrant ligands will provide further therapeutic avenues through greater reward system activation.

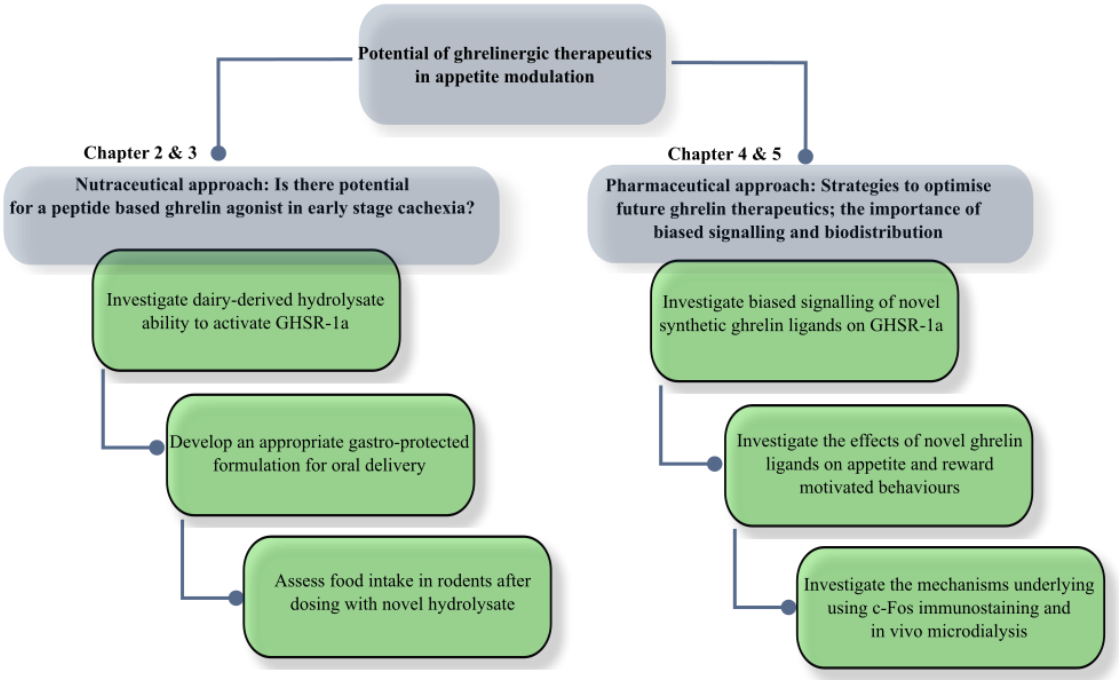


***Figure 1: Thesis summary.*** *Flow chart of overall aims of thesis.*

# Chapter 1

*Adapted from*

# From Belly to Brain: Targeting the Ghrelin Receptor in Appetite and Food Intake Regulation

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## Evolutional perspectives on energy balance

A fundamental requirement for survival of an organism is the appropriate maintenance of energy balance. Times of food deprivation require adequate storage of fat to utilize as an energy substrate, while times of food surplus often require overconsumption to replenish energy stores. The basic need for this energy conservation and replacement, as well as energy-expensive demands such as foraging for food, flight from predators, reproduction etc., require an effective metabolic machinery to maintain homeostasis. In the short term, this machinery must be capable of dealing with the circadian oscillations in energy intake e.g. fasting during sleep, to a longer-term cycle of famine that was synonymous with the lifestyle of our ancestral hunter-gatherers, and many other eras throughout history (Berbesque, Marlowe et al. 2014). A malfunctioning internal milieu for energy balance confers a distinct disadvantage to our forebears who endured famine – indeed, those of us alive today are likely to have descended from evolution's selection of those who are genetically geared to efficiently store energy during times of famine (Waterson and Horvath 2015).

However, the evolutionary drive to overconsume calories to compensate for periods of famine has become a redundant trait in the last century. The brain has evolved over millennia to promote the motivation to obtain food in order to enhance survival – nowadays, however, it is wired inappropriately to deal with the surplus of readily available energy in the Western world (Neel 1962). This results in the overconsumption of food and surplus in calories compared to metabolic requirements, and subsequent fat accumulation (Zheng, Lenard et al. 2009). In this respect, it is no surprise that the abundance of food, particularly high-calorie convenience food available to us in the Western world has resulted in an obesity crises of epidemic proportions (Wyatt, Winters et al. 2006).

On the converse, dysregulation of these fundamental mechanisms for energy balance are seen in conditions of undereating and illness-associated weight loss, where appetite and motivation to seek out food is very low (Morley, Thomas et al. 2006). A natural decline in appetite and food intake occurs in elderly populations – this can lead

to malnutrition with a reduction in immunity, energy levels, independent living and overall health (Chapman 2004, Hickson 2006, Thomas 2007, Malafarina, Uriz-Otano et al. 2013). The reasons behind decreases in appetite result from a combination of physiological changes, which lead to earlier satiation and a decreased ability of the body to regulate energy balance (Chapman 2004, Hickson 2006). Furthermore, changes to sensory system, cognitive and emotional processes lead to a reduced incentive valuation placed on food (Jacobson, Green et al. 2017). Complicating this, ageing population demographics lead to an increased prevalence of chronic illnesses, which compound a weakening metabolism (Organization 2015). Subsequently, this can result in the wasting syndrome known as cachexia, which is a complex metabolic syndrome accompanied by illness resulting in intractable loss of weight and a poorer prognosis for the accompanying illness (Morley, Thomas et al. 2006, Evans, Morley et al. 2008).

The neural circuits controlling energy metabolism are critical junctures for the successful treatment of conditions of both over-eating and under-eating (Gautron, Elmquist et al. 2015, Waterson and Horvath 2015). The mechanisms underlying food intake have been under scrutiny for decades, with particular advances being made since the late 1990's and the discovery of ghrelin. However, a lot remains to be uncovered in the neural control of appetite, while the search for successful appetite modulation strategies to harness these evolved mechanisms is ongoing.

## 1.1 Neural basis of energy homeostasis

Research has revealed that food intake is one of the most deceptively variable and complex of mammalian behaviours. Daily food intake varies both between and within individuals – basal energy requirements are dictated by calories consumed and expended, while more subjective factors such as social conformity, stress levels, cost, convenience, perceived palatability etc. all ultimately feed into the decision to eat in any given situation (Waterson and Horvath 2015). One of the most recognized explanations of appetite control is the energy balance theory, although recent years have led to some criticism of this. While there may be limitations, this "set-point" theory is probably the most influential and argued theory of body weight control to

date. This theory is based on the premise that a delicate homeostatic "set-point" for body weight is maintained, from which body weight can fluctuate slightly with eating behaviours. However, the reality is that the plethora of factors which impact on our ultimate appetite levels is highly complex and variable. The mechanisms underpinning our drive to consume food have become increasingly known since the turn of the century. In more recent years, the "set-point" theory has been largely overshadowed by the neural basis of appetite regulation, which has become widely known (Harris 1990, Waterson and Horvath 2015). Specific neuronal populations have been identified which are responsible for enhancing hunger and satiety. Furthermore, there are neuronal highways which connect these areas to the pleasure centres of the brain which can affect our perception of food, or the motivation to obtain it.

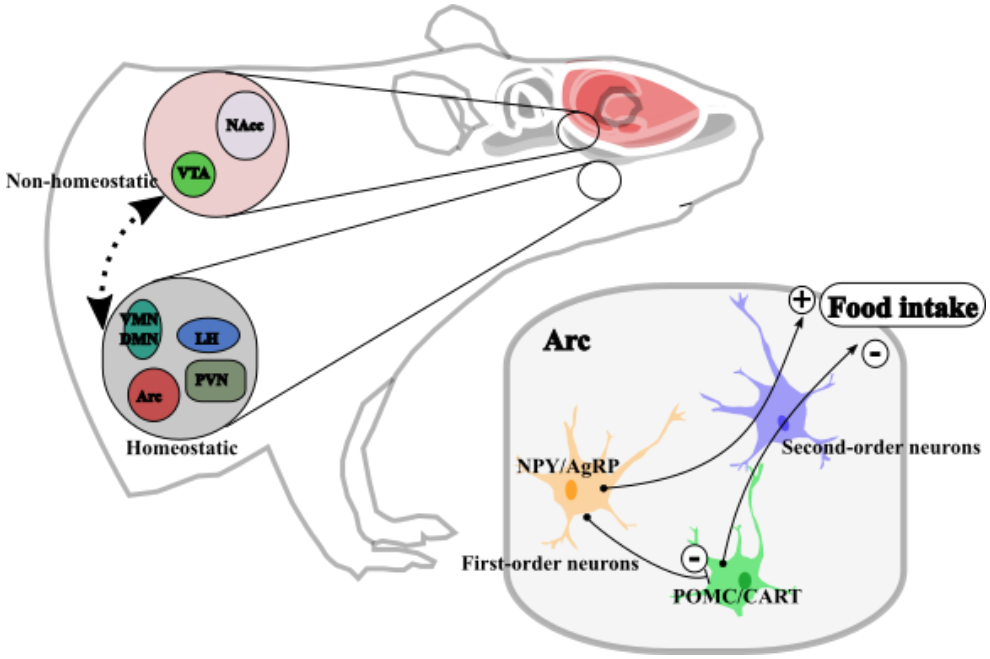



***Figure 1.1. Neural basis of appetite and food reward.*** *Schematic representation of two overlapping areas of appetite and food intake; homeostatic food intake is regulated through the hypothalamus, while non-homeostatic largely feeds into the reward circuitry. Abbreviations: Arc; arcuate nucleus, PVN; paraventricular nucleus, LH; lateral hypothalamus, V/DMN; ventral/dorsomedial nucleus, VTA; ventral tegmental area; NAcc; nucleus accumbens, NPY; neuropeptide Y, AgRP; agouti-related peptide, POMC; pro-opiomelanocortin, CART; cocaine and amphetamine regulated transport.*

The neural basis of appetite and food intake can for simplicity be subdivided into two distinct yet overlapping areas of *homeostatic* and *non-homeostatic* (pleasurable or hedonic) feeding (Figure 1.1). The primary fulcrum for *homeostatic* neuronal control of appetite is the hypothalamus. The mammalian hypothalamus is a forebrain structure which can be divided into 40 different sub nuclei, and is associated with various behaviours, including drinking, sexual behaviour, aggression, as well as body temperature regulation and immunity (Berthoud 2002). Critically, it is the key structure responsible for the regulation of food intake, energy balance and fat storage. The strategic location of the hypothalamus within the mammalian neuroendocrine machinery means that it receives a wealth of neurohumoral input, thereby allowing it to gain information on the central and peripheral state of affairs with regard to energy state (presence of satiating or hunger factors in the blood), and the availability of food (olfactory, visual, gustatory etc.), as well as many other inputs such as stress, fight or flight response etc. Three key hypothalamic substructures relating to food intake are the arcuate nucleus, the lateral hypothalamus and the paraventricular hypothalamus.

The arcuate nucleus (Arc) is in an ideal location to receive a wealth of information regarding energy balance. Leptin, produced from adipose tissue, provides humoral information about long-term energy stores (Klok, Jakobsdottir et al. 2007), while more short-term information is obtained from plasma levels of hormones related to meal-intake (e.g. insulin, ghrelin) and from glucose-sensing neurons (Grossman 1986). Furthermore, signals are conveyed from the gut to the hypothalamus by way of the vagus nerve, an information highway between the gut and the brain (Sawchenko 1983). Top-down information from neurons in various cortical areas, amygdala, and bed nucleus of the stria terminalis convey immediate visual, gustatory and olfactory information, as well as reward expectancies, learned behaviours and emotional aspects of particular foods (Berthoud 2002). In turn, the Arc neurons have reciprocal connections to all of these areas, many of these via the lateral hypothalamus (LH) (Berthoud 2002).

The LH is another key hypothalamic substructure, which although lacking a strong direct endocrine output due to its spatial separation from the median eminence (ME), has an impressive array of output connections to the telencephalon, hindbrain

and spinal cord, enabling it to engage both the skeletal (behavioural) and autonomic output systems. Of these outputs, melanocortin hormone (MCH) and orexin neurons play significant roles in feeding and energy balance. In turn, the LH receives direct and indirect sensory signals from various hypothalamic, cortical and limbic structures ranging from olfactory, gustatory and visual input, to mechanical information from the gut (Simerley 1995, Rempel-Clower and Barbas 1998) (Berthoud 2002).

Overall, these areas of the hypothalamus along with many others (not described here for brevity) play pivotal roles in the regulation of energy balance. The neural mechanisms of energy balance involve an intricate balance of communication and feedback between the various hypothalamic nuclei and an information highway with hormonal, metabolite and neural traffic. This dynamic internal picture is then relayed to endocrine and autonomic effectors to complete a complex, fluid feedback loop.

However, the hypothalamus-regulated homeostatic regulation of appetite and food intake is only one part of the appetitive framework. Non-homeostatic neural mechanisms are also an important consideration in the overall control of food intake (Berthoud 2006, Wise 2006, Egecioglu, Skibicka et al. 2011, Waterson and Horvath 2015). The term "non-homeostatic" encompasses both motivation and incentive salience applied to food rewards, but also the inherent palatability or "hedonic" aspect of eating in itself. These rewarding properties of eating, beyond metabolic demand are largely controlled by the mesolimbic reward system (Wise 2006). The reward system circuitry is comprised of the ventral tegmental area (VTA) and its main projection site, the nucleus accumbens (NAcc), a hotspot for dopamine (DA) release (Swanson 1982, Bassareo and Di Chiara 1999, Spanagel and Weiss 1999). This pathway is critical to the motivation to seek-out and obtain a rewarding stimulus, and is associated with promoting incentive valuation of drugs of abuse, as well as natural rewards, including food (Kenny 2011, Volkow, Wang et al. 2012).

Although spatially and functionally separated from the hypothalamus, there is a lot of overlap between the mesolimbic reward circuitry and the neural pathways involved in homeostatic food intake (Berthoud 2006, Volkow, Wang et al. 2011,

Howick, Griffin et al. 2017). Indeed, almost any food tastes better when hungry compared to a state of satiation (Perello and Dickson 2015). Orexin and glutamatergic neurons from the LH activate dopaminergic neurons in the VTA, the main projection site to the NAcc. Indeed, VTA activation by peripheral ghrelin is dependent on functional orexin neurons in the LH (Perello, Sakata et al. 2010). By contrast, the hypothalamic effect on food intake is dependent on the reward circuitry to effect the intended modification to food intake. Therefore, it seems that the so-called homeostatic and non-homeostatic feeding behaviours are intertwined (Volkow, Wang et al. 2011).

In summary, the presence or perception of hunger in a given situation is a net result of a dynamic balance of ascending neurohumoral feedback on energy status, as well as sensory information from higher brain areas associated with reward and motivation. The top-down regulation of food intake and the underlying neural mechanisms are extremely complex, and although a lot is now known, a lot is yet to be discovered in this area. The vast array of communicating neuropeptides and the volume of neuronal communication between the various sub-regions of the hypothalamus, telencephalon and brainstem is a testament to this very complicated picture (Berthoud 2002). One of the most investigated hormones orchestrating many changes to these areas is the peptide hormone and neuropeptide, ghrelin.

## Ghrelin and the GHSR-1a in appetite and food intake regulation

The ghrelin hormone is the only known peripherally produced orexigen. The 28aa peptide was discovered by Kojima and colleagues in 1999, is synthesized by gastric endocrine cells (Kojima et al., 1999). Initially, ghrelin was discovered to be the endogenous ligand for the GHSR-1a, responsible for eliciting growth hormone (GH) release from the anterior pituitary gland. Matthias Tschöp and colleagues reported soon after that ghrelin was responsible for regulating food intake, body weight, adiposity and glucose metabolism (Tschop, Smiley et al. 2000). Due to its proximal relationship with mealtimes, spiking pre-prandially and returning to baseline in the

post-ingestive phase, ghrelin was originally known as a “meal initiation” or “hunger” hormone (Cummings, Purnell et al. 2001). It was postulated to act as a gastrointestinal signal for fuel status to the brain, adjusting food intake and energy expenditure (Inui 2001, Asakawa, Inui et al. 2005). Consistent with this notion, the oxyntic cells of the stomach release ghrelin into the bloodstream (Inui 2001) when hunger is perceived (Cummings, Purnell et al. 2001). Subsequent hypothalamic GHSR-1a-mediated activation of arcuate nucleus neuropeptide Y/agouti-related peptide neurons serves to stimulate orexigenic activity through Y1 receptors, while concomitantly inhibiting satiating pro-opiomelanocortin neurons (Nakazato, Murakami et al. 2001, Cowley, Smith et al. 2003). Furthermore, ghrelin contributes to the regulation of body weight by potently stimulating GH secretion from the pituitary, increasing adiposity and reducing energy expenditure (Takaya, Ariyasu et al. 2000, Wren, Small et al. 2000). Recently, however, this traditional and narrowly defined view of ghrelin as a “hunger hormone” has been challenged (McFarlane, Brown et al. 2014).

Increasing evidence supports a more complex role for ghrelin in the regulation of hunger and metabolism. Goldstein and Brown showed that ghrelin-stimulated GH secretion is critical to protecting the body from starvation-induced hypoglycaemia (Goldstein, Zhao et al. 2011). Ghrelin is also implicated as a contributor in reward processing, memory consolidation, response to stress, gastrointestinal motility, glucose homeostasis and many other functions (Masuda, Tanaka et al. 2000, Carlini, Monzon et al. 2002, Abizaid, Liu et al. 2006, Chuang, Perello et al. 2011).

## 1.2 Ghrelin - Production, Cleavage and Octanoylation

Ghrelin is encoded by the ghrelin gene (ghrl) in humans, located on chromosome 3p25-26 from which alternative splicing and post translational modification can yield a variety of bioactive molecules such as obestatin and des-acyl ghrelin (Figure 1.2) (Zhang, Ren et al. 2005). Ghrelin is derived from enzymatic cleavage of preproghrelin with ghrelin-o-acetyl transferase (GOAT) (Gualillo, Lago et al. 2008), an enzyme which activates the peptide via n-octanoylation on the serine 3 residue, yielding acyl-ghrelin (Kojima, Hosoda et al. 1999). Acylated and des-acylated found in the circulation, however only the acylated form acts as a modulator

of the GHSR-1a (Bednarek, Feighner et al. 2000). Des-acylated ghrelin is the most abundant from found in the circulation and is suggested to be the active ligand for an additional, unidentified, subtype of the GHSR. There is also increasing evidence pointing to the importance of des-acyl ghrelin as a distinct, pharmacologically active moiety, rather than an inactive, neutral entity (Inhoff, Monnikes et al. 2008, Delporte 2013). Indeed, recent evidence has begun to unravel various effects of desacyl-ghrelin on food intake and gastric motility. The reader is directed to the latter part of this introduction (Section 1.7.1) for more information here.

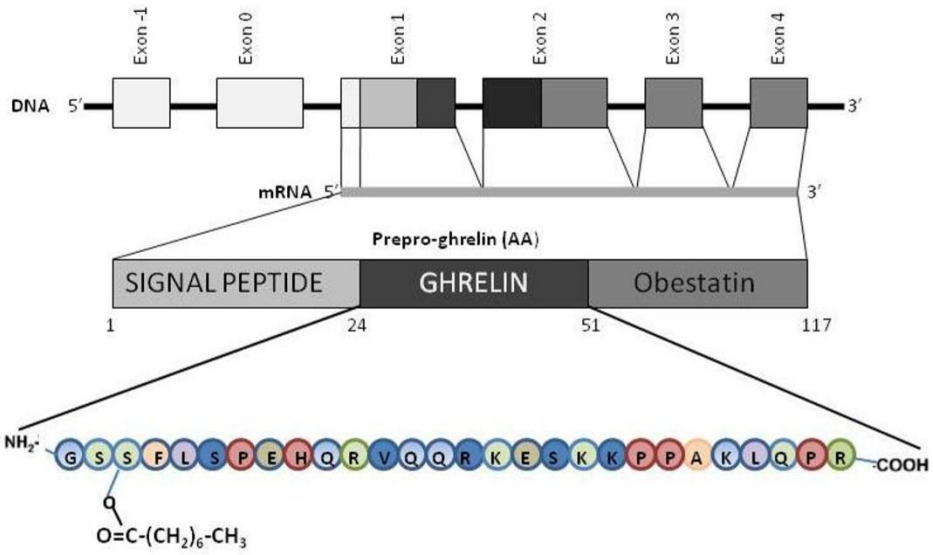



***Figure 1.2. Production and Cleavage of Acyl-ghrelin:*** *Ghrelin is encoded by the ghrelin gene (ghrl) in humans, located on chromosome 3p25-26, and enzymatic cleavage of preproghrelin with ghrelin-o-acetyl transferase (GOAT) activates the peptide via n-octanoylation on the serine 3 residue, yielding acyl-ghrelin. Figure reproduced from Schellekens et. al (Schellekens, Dinan et al. 2013)*

## 1.3 Role of ghrelin in homeostatic and non-homeostatic feeding

Like appetite and food intake, ghrelin's role can be subdivided into the two mutually dependent categories of homeostatic and non-homeostatic feeding (Berthoud 2006, Jerlhag, Egecioglu et al. 2006, Dickson, Egecioglu et al. 2011, Egecioglu, Skibicka et al. 2011, Schellekens, Dinan et al. 2013). The term "non-homeostatic" encompasses both motivation and incentive salience applied to food rewards, but also the inherent palatability or "hedonic" aspect of eating in itself. The ghrelin system not only acts as a barometer for energy balance (Tschop, Smiley et al. 2000, Nakazato, Murakami et al. 2001), but also contributes to the drive for eating beyond metabolic demand and the consumption of palatable foods (Dickson, Egecioglu et al. 2011, Egecioglu, Skibicka et al. 2011). Therefore, ghrelin and the GHSR-1a, have been extensively investigated as potential therapeutic targets to tackle metabolic, eating- and appetite-related disorders by virtue of the unique position which the ghrelinergic system occupies at the interface of homeostatic and hedonic feeding (Lutter and Nestler 2009, Schellekens, Finger et al. 2012, Schellekens, Dinan et al. 2013, Perello and Dickson 2015).

The ghrelinergic system has received considerable focus as a target in maladaptive changes to homeostatic energy balance (Tschop, Smiley et al. 2000, Cummings 2006, Argilés, López-Soriano et al. 2008). This is achieved through manipulating a number of physiological mechanisms resulting in a net anabolic effect in the body (Cowley, Smith et al. 2003, De Vriese, Perret et al. 2010). The normal ageing process yields a number of physiological changes, which lead to a reduction in appetite and appropriate nutritional intake (Chapman 2004, Malafarina, Uriz-Otano et al. 2013). Declining ghrelin levels contribute to this reduction in food intake and lean body mass (Malafarina, Uriz-Otano et al. 2013). Furthermore, ageing population demographics translate to a greater incidence of chronic conditions such as cardiovascular disease, respiratory disease and cancer (Organization 2015). Chronic diseases compound a weakening ghrelin axis by increasing systemic inflammation and cytokine output (DeBoer 2008). Cytokine-mediated activation of anorexigenic neuron populations in the hypothalamus causes a cascade of metabolic changes resulting in loss of lean and fat mass, and the development of cachexia (Chapman 2004, DeBoer

2011, Malafarina, Uriz-Otano et al. 2013). Thus, a metabolic backdrop is created which antagonises ghrelin's somatotrophic effect (DeBoer 2008, DeBoer 2011, Nass, Gaylinn et al. 2011). Age-related malnutrition and under-eating following chronic diseases results in prolonged hospital stays, decreased independence and poorer response to treatment, leading to a greater burden on global health infrastructures and poorer clinical outcomes (Chapman 2004, Hickson 2006, Malafarina, Uriz-Otano et al. 2013).

Further to its role as a key mediator of the energy balance "set point", ghrelin is also implicated in incentive salience and motivation to eat, and consequently has become a therapeutic target for development of therapies for overeating and obesity (Wren, Small et al. 2001, Horvath, Castaneda et al. 2003). The need for anti-obesity therapeutics is highlighted by the global increase in incidence of obesity in recent years. In 2014, more than 1.9 billion adults (39% globally) were overweight (WHO 2016) and obesity continues to rise to epidemic proportions. In Western society particularly, consumption of readily available high-fat and high-sugar meals, together with increasingly sedentary lifestyles has led to a rise in the "metabolic syndrome". This is a condition associated with weight gain, hyperglycaemia, insulin resistance, hypercholesterolaemia and a general inflammatory phenotype (Isomaa, Almgren et al. 2001, Martin, Mani et al. 2015). In addition to homeostasis, neuronal pathways also exist which promote the consumption of palatable, calorie-dense foods beyond the metabolic demands of the organism (Kenny 2011). This is thought to be an evolutional mechanism that promotes over-eating of calorie-dense foods in preparation for times of food deprivation. This is redundant in the Western world where there is an abundance of food. The mesolimbic dopaminergic pathway in the brain is known to be a key mediator in this primitive drive (Spanagel and Weiss 1999, Pierce and Kumaresan 2006, Volkow, Wang et al. 2012). Overconsumption of palatable foods is thought to be triggered by hyperactivity of the reward system (Stoeckel, Weller et al. 2008, Stice, Yokum et al. 2010). Furthermore, the late Bart Hoebel and colleagues in Princeton proved that sugar in itself can share many of the properties of addictive substances (Konturek, Konturek et al. 2004, Avena, Rada et al. 2008). In fact, palatable foods are now known to share the same reward pathways as non-psychostimulant drugs of abuse (Tanda and Di Chiara 1998). It should be noted that although the

concept of food addiction has gained significant ground, it has many heuristic limitations (Volkow, Wang et al. 2013, Hebebrand, Albayrak et al. 2014).

Increases in circulating levels of endogenous ghrelin, following periods of food restriction, signal an increase in appetite and hunger and are correlated with a general increase in both "liking" and "wanting" of food (Druce, Wren et al. 2005, Perello and Dickson 2015). Interestingly, the elevated endogenous ghrelin levels have been associated with an increased DA output in the brain (Kawahara, Kaneko et al. 2013), while functional magnetic resonance imaging in human subjects has shown that ghrelin administration enhances the activation of the central reward circuitry in response to images of pleasurable foods (Malik, McGlone et al. 2008, Goldstone, Prechtl et al. 2014). Subsequently, ghrelin's role in increasing the incentive valuation of food at the level of the mesolimbic circuitry has come to the fore in recent reviews (Andrews 2011, Perello and Dickson 2015).

## 1.4 GHSR-1a – Biodistribution and Signalling

The target for ghrelin and ghrelin ligands is the GHSR-1a, a 7 transmembrane G-protein coupled receptor (GPCR). The GHSR-1a is expressed both in the central nervous system (CNS) and peripherally in the body, and binding of acyl-ghrelin leads to receptor activation (Kojima, Hosoda et al. 1999). The distribution of the GHSR-1a is of paramount importance as it is the executor of ghrelin's function. Indeed, it is the peripheral (exclusive to non-CNS tissue) and central (exclusive to the CNS) distribution of the GHSR-1a which is responsible for the plethora of physiological effects which ghrelin exerts (Figure 1.3)(Howick, Griffin et al. 2017). The GHSR-1a is densely expressed in the hypothalamic nuclei which sends neuronal projections to other appetite regulating centres (Gnanapavan, Kola et al. 2002, Andrews 2011). Peripherally, GHSR-1a is located on vagal afferents, pancreatic cells, spleen, cardiac muscle, bone, adipose, thyroid, adrenal glands and on immune cells (Gnanapavan, Kola et al. 2002, Stengel, Goebel et al. 2010). Therefore, given the ubiquitous expression of the receptor, any instance of exogenous ghrelin or ghrelin ligand administration leads to a combination of downstream effects. Neither exogenous ghrelin nor ghrelinergic compounds can effectively target centrally-controlled food

intake, without affecting a multitude of other central and peripheral outputs (Horvath, Castaneda et al. 2003, Müller, Nogueiras et al. 2015). The non-specific tissue effects of peripheral ghrelin administration may be further complicating an intricate metabolic balance and need to be considered.

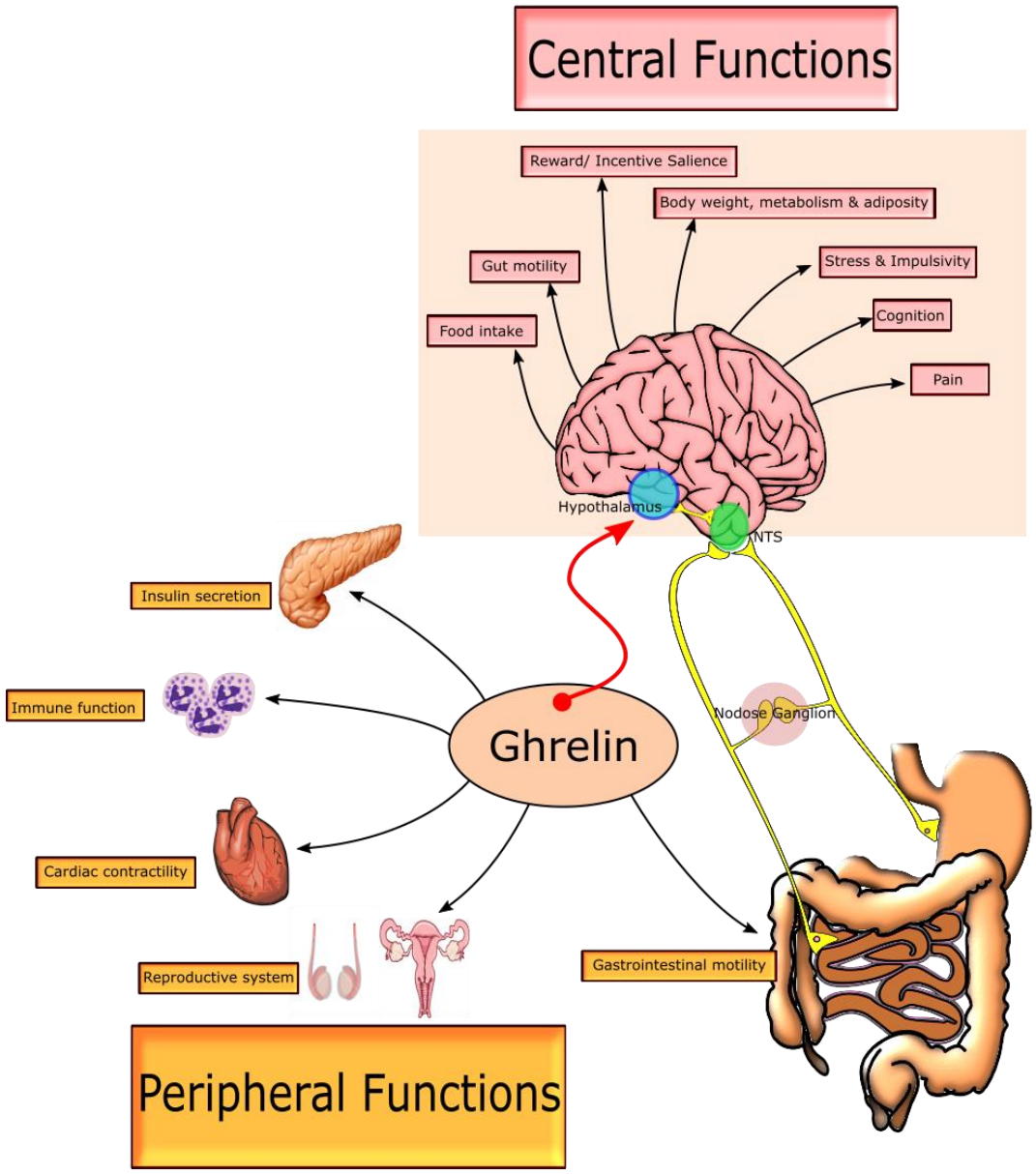



***Figure 1.3. Central and Peripheral functions of ghrelin.*** *This combines the documented methods of ghrelin's action after its release from the stomach, or exogenous administration. Ghrelin travels via the circulation to activate the growth hormone secretagogue receptor (GHSR-1a) in the arcuate nucleus (Arc) and the nucleus tractus solitarius (NTS) after circumventing the blood-brain barrier (BBB), denoted by the red arrow. Peripheral signals are conveyed to the central nervous*

*system (CNS) via vagal afferents also. Activation of the GHSR-1a leads to a multitude of centrally and/or peripherally mediated effects*

### 1.4.1 Central Ghrelinergic Signalling

Food intake, adiposity and energy homeostasis are centrally controlled functions of ghrelin and the GHSR-1a which have been extensively described in the literature (Tschop, Smiley et al. 2000, Nakazato, Murakami et al. 2001, Cowley, Smith et al. 2003). Chronic central administration of ghrelin induces adiposity in rodents by reducing the utilization of fat as an energy substrate (Tschop, Smiley et al. 2000). Further work confirmed this central action, with expression of mRNA for fat-sparing enzymes fatty-acid synthase, acetyl-CoA carboxylase α, stearoyl-CoA desaturase-1, and lipoprotein lipase all being increased with chronic intracerebroventricular infusion of ghrelin. In addition, mRNA expression for carnitine palmitoyltransferase-1α, involved in fat utilisation is decreased while lipid mobilization is reduced following ghrelin treatment, as shown by an increase in respiratory exchange ratio *in vivo* (Theander-Carrillo, Wiedmer et al. 2006, Davies, Kotokorpi et al. 2009). Furthermore, ghrelin stimulates lipid deposition in human visceral adipose tissue in a dose-dependent manner (Rodriguez, Gomez-Ambrosi et al. 2009). Acute ghrelin administration consistently stimulates food intake across species (Wren, Small et al. 2000, Cummings, Purnell et al. 2001, Nagaya, Uematsu et al. 2001, Wren, Small et al. 2001, Mericq, Cassorla et al. 2003, Chen, Trumbauer et al. 2004, Druce, Wren et al. 2005, Schmid, Held et al. 2005, Wynne, Giannitsopoulou et al. 2005). In recent years however, research has proven that ghrelin may not be the critical regulator of food intake it was once heralded to be.

Studies in knockout mice have confirmed the ghrelin peptide is not a key mediator of food intake or growth (Sun, Ahmed et al. 2003). In contrast with predictions, ghrelin knockout mice are neither undersized nor hypophagic; their behavioural phenotype for food intake and physical attributes are indistinguishable from wild-type littermates (Sun, Ahmed et al. 2003, McFarlane, Brown et al. 2014). Ghrelin-null rodents also display normal responses to starvation and diet-induced obesity (Sun, Ahmed et al. 2003). Furthermore, ablation of ghrelin in adulthood failed to elicit effects on food intake, body weight, or resistance to diet-induced obesity

(McFarlane, Brown et al. 2014). Interestingly, both germline ghrelin-deficient and ghrelin cell-ablated mice display a profound hypoglycaemia following prolonged calorie restriction. Overall however, the phenotype in ghrelin-knockouts is suggestive of a non-critical role for ghrelin in food intake and growth.

Despite the apparent compensatory mechanisms that exist in the absence of ghrelin, exogenous ghrelin or ghrelin ligands have the potential to significantly modulate appetite, most likely via central GHSR-1a signalling. Recently it was shown through neuronal-specific ablation of the GHSR-1a that receptor signalling within the CNS is a crucial regulator of energy metabolism. This is important to consider in the context of the high constitutive activity of the GHSR-1a, which does not require ghrelin in order to become activated (Holst and Schwartz 2004, Petersen, Woldbye et al. 2009). Zigman and colleagues, amongst others, have demonstrated that GHSR-1a-null mice are resistant to diet-induced obesity (Zigman, Nakano et al. 2005, Ma, Lin et al. 2011, Lin, Lee et al. 2014). Neuronal GHSR-1a is also essential for ghrelin-induced meal initiation and maintenance of body weight in conditions of caloric deficit (Lee, Lin et al. 2016). Central GHSR-1a signalling therefore seems to be critical for not only acute initiation of food intake, but also is a key mediator of body weight. Supporting this, a genetic mutation in GHSR-1a that allows ghrelin binding but prevents activation of the receptor, leads to the condition of familial short stature (Pantel, Legendre et al. 2006).

Consistent with the notion of a multifunctional role for ghrelin, the GHS-R1a is also expressed in several non-hypothalamic brain areas. *In-situ* binding studies have demonstrated the existence of the GHSR-1a in the midbrain DA system, particularly the main mesolimbic reward circuitry structures; the ventral tegmental area (VTA) and its primary projection site, the NAcc (Abizaid, Liu et al. 2006, Zigman, Jones et al. 2006, Landgren, Simms et al. 2011). The VTA projects GHSR-1a-expressing dopaminergic neurons which terminate in the NAcc, a hotspot for DA release which is critically associated with promoting incentive value of drugs of abuse and natural rewards, including food (Liu and Borgland 2015). Further projections from the VTA to the medial prefrontal cortex, an important part of the reward system which also encodes the genes for the GHSR-1a, are described as part of this pathway (Swanson

1982, Tzschentke 2000, Landgren, Engel et al. 2011). Consequently, the GHSR-1a located in the midbrain dopaminergic pathway may be a driver for the decision to eat palatable, calorie-dense foods, irrespective of metabolic need.

GHSR-1a is also expressed in areas associated with memory, emotional arousal and cue-potentiated feeding (Diano, Farr et al. 2006, Kern, Mavrikaki et al. 2015, Müller, Nogueiras et al.). For example, GHSR-1a in the hippocampus is known to play a role in synaptic plasticity, increasing hippocampal spine density and enhancing long-term potentiation, an important phenomenon in learning and memory consolidation (Diano, Farr et al. 2006). Activation of hippocampal GHSR-1a *in vivo* increased performance and retention of memory-dependent tasks (Carlini, Monzon et al. 2002, Diano, Farr et al. 2006). Furthermore, the GHSR-1a is densely expressed in several sub-nuclei of the amygdala and is associated with amelioration of anxiety-like behaviours in food scarcity (Alvarez-Crespo, Skibicka et al. 2012). Altogether, the above is supportive of a broader, non-homeostatic function for GHSR-1a signalling in higher brain functions dependent on metabolic status, for example, heightened salience and increased memory consolidation in times of hunger to remember where food can be obtained (Diano, Farr et al. 2006). Critically, although ghrelin peptide mRNA is not found in the brain, it's expression is noted peripherally, suggesting multiple potential autocrine or paracrine roles of the hormone (Gnanapavan, Kola et al. 2002, Sakata, Nakano et al. 2009, Furness, Hunne et al. 2011). Indeed, direct actions of ghrelin in the periphery have been reported in several organ systems.

### 1.4.2 Peripheral Ghrelinergic Signalling

The GHSR-1a is responsible for several peripheral mechanisms modulated by ghrelin including, but not limited to, cardiac contractility, bone formation and reproductive function. Firstly, GHSR-1a is expressed on rodent and human immune cells, including monocytes and T cells (Gnanapavan, Kola et al. 2002, Dixit, Schaffer et al. 2004). Ghrelin and ghrelin agonists have shown a protective effect under acute endotoxaemia, enhancing the effectiveness of immune response through tissue infiltration *in vivo* (Chen, Liu et al. 2008, Li, Li et al. 2010), leading to decreased mortality. Ghrelin is also known to directly reduce the expression of inflammatory

cytokines (Dixit, Schaffer et al. 2004). Secondly, protective effects have also been attributed to ghrelin in rodent cardiomyocytes (Baldanzi, Filigheddu et al. 2002, Lear, Iglesias et al. 2010). The cardioprotective mechanisms underlying this have been described in detail elsewhere (Pang, Xu et al. 2004). The ghrelin agonist, hexarelin, was shown to increase cardiac output in rodents and humans (Bisi, Podio et al. 1999, Nagaya, Uematsu et al. 2001). Thirdly, ghrelin and the GHSR-1a are expressed in rat and human testis (Barreiro, Gaytan et al. 2002, Gnanapavan, Kola et al. 2002, Gaytan, Barreiro et al. 2004) and in females both have been documented to be expressed in ovary, hilus cells (leydig cells) and corpora lutea, all of which are hormone secreting cells which play roles in the female reproductive cycle (Muccioli, Lorenzi et al. 2011). Ghrelin plays a crucial role in the regulation of the hypothalamic-pituitary-gonadal axis mainly through reducing secretion of hypothalamic gonadotropin-releasing hormone and stimulating local luteinizing hormone and follicle stimulating hormone secretion.

### 1.4.3 Complementary Ghrelinergic Signalling: Gastrointestinal Motility, Glucose Homeostasis and Visceral Pain

All of the above have discussed distinct centrally-mediated and non-central autocrine or paracrine functions of GHSR-1a. In certain instances, central and peripheral ghrelinergic signalling appear to be complementary, as is the case for regulation of gastrointestinal motility, glucose homeostasis and visceral pain. The role of ghrelin and the GHSR-1a in the regulation of gastrointestinal tract motility has already been reviewed (De Smet, Mitselos et al. 2009). The GHSR-1a is located in the mucosa and myenteric plexus of rodent and human gastrointestinal tract, reinforcing the local neural role for ghrelin in gut motility (Date, Murakami et al. 2002, Dass, Munonyara et al. 2003, Takeshita, Matsuura et al. 2006). *In vitro*, this notion was supported by contractility studies showing that ghrelin directly activates both cholinergic (Dass, Munonyara et al. 2003, Fukuda, Mizuta et al. 2004, Depoortere, De Winter et al. 2005) and tachykinergic excitatory neurons in fundus and antrum. *In vivo,* peripheral administration of ghrelin accelerates gastric emptying in a dose-dependent

manner (Trudel, Tomasetto et al. 2002, Depoortere, De Winter et al. 2005, Dornonville de la Cour, Lindqvist et al. 2005, Kitazawa, De Smet et al. 2005). In humans, ghrelin infusion stimulates gastric emptying in healthy participants and ameliorates symptoms of gastroparesis (Levin, Edholm et al. 2006). However, central administration also displays a pronounced effect on gastrointestinal tract motility (Asakawa, Inui et al. 2001, Fujino, Inui et al. 2003). Vagotomy or chemical deactivation of the vagus were shown to abolish the observed effects of peripherally administered ghrelin (Masuda, Tanaka et al. 2000, Fukuda, Mizuta et al. 2004). Ghrelin's effects in respect of gastrointestinal motility thus seem to be vago-vagal in origin—meaning that it results from reciprocal vagal communication between the gut and the dorsal vagal complex of the brain. Similar to food intake and adiposity above, gastric emptying is unaffected in ghrelin knockout rodents, suggesting the existence of compensatory mechanisms (De Smet, Mitselos et al. 2009). Critically, it has been suggested that local mechanisms become operational under abnormal conditions such as vagal denervation or pharmacological stimulation (Fujino, Inui et al. 2003). Supporting this, it was shown that downregulation of GHSR-1a in the small intestine delays transit in vagotomised mice (Yang, Qiu et al. 2011). Overall, evidence suggests that ghrelin acts from the periphery in a remote fashion to modulate gastrointestinal function from the CNS via the vagus nerve, however the gastrointestinal distribution of the GHSR-1a paves the way for local activity which may be heightened by pharmacological stimulation (Fujino, Inui et al. 2003). The motilin receptor has also been characterized in the human gastrointestinal tract (Feighner, Tan et al. 1999) and displays close structural homology and a functional compensatory role with the GHSR-1a in gastrointestinal motility (Nunoi, Matsuura et al. 2012).

Interacting central and peripheral GHSR-1a signalling is evident in the physiology of glucose homeostasis. Many peripheral hormones act in a central manner to regulate energy metabolism and glucose balance, including glucagon, glucagon-like peptide 1 and insulin (Obici, Zhang et al. 2002, Sandoval, Bagnol et al. 2008, Morton and Schwartz 2011, Mighiu, Yue et al. 2013). However, the GHSR-1a is expressed in pancreatic α and β cells (Date, Murakami et al. 2002, Date, Nakazato et al. 2002, Dezaki, Hosoda et al. 2004, Kageyama, Funahashi et al. 2005), and peripheral ghrelin acts directly on the receptor in pancreatic islets to modulate the release of insulin

(Reimer, Pacini et al. 2003, Dezaki, Hosoda et al. 2004, Tong, Prigeon et al. 2010). In humans, Broglio and colleagues found that acute administration of acyl-ghrelin in the fasted state significantly reduced plasma insulin while promoting hyperglycaemia, however, a continuous infusion stimulated insulin secretion secondary to elevated glucose levels (Broglio, Arvat et al. 2001, Broglio, Prodam et al. 2008). Supporting this, several studies have consistently shown that ghrelin administration promotes hyperglycaemia (Garin, Burns et al. 2013). Central administration of ghrelin also regulates plasma insulin in rodents (Kim, Namkoong et al. 2004, Nesic, Stevanovic et al. 2008, Heppner, Piechowski et al. 2014, Stark, Reichenbach et al. 2015). Somewhat confusingly, it seems that central GHSR-1a signalling exerts an insulinotropic effect, versus the inhibition of glucose-stimulated insulin secretion by peripheral GHSR-1a activation (Tong, Prigeon et al. 2010, Heppner, Piechowski et al. 2014), meaning that the receptor may play distinct roles in glucose homeostasis depending on the site of action. Furthermore, administration of acyl-ghrelin into the portal, but not the femoral vein inhibited glucose-stimulated insulin secretion. Hepatic vagotomy attenuated this inhibition suggesting indirect central control over insulin secretion via neural signalling (Cui, Ohnuma et al. 2008, Meyer 2010). Critically, fasting decreases insulin levels in both wild type and ghrelin knockouts, as well as producing comparable responses to both hypo-caloric and hyper-caloric situations. Hence, compensatory pathways seem to exist for glucose homeostasis, however GHSR-1a knockout leads to reduced glucose levels under calorie- deprivation (Sun, Ahmed et al. 2003, Sun, Butte et al. 2008). Later work from the same group used GHSR-1a-null mice to show reduced adiposity and insulin resistance (Lin, Saha et al. 2011). Thus, a body of evidence exists to support the indirect central control of GHSR-1a signalling over glucose homeostasis. Furthermore, it seems that metabolic status is a key determinant of the regulatory action of central ghrelin on peripheral glucose homeostasis (Stark, Reichenbach et al. 2015). A recent review summarized the complex interrelationship that exists between ghrelin, insulin and glucose (Chabot, Caron et al. 2014). The ability of insulin and glucose levels to appreciably impact on appetite (Woods, Lutz et al. 2006) means that indiscriminate targeting of the GHSR-1a without due consideration of the effects on peripheral glucose and insulin metabolism may ultimately decrease

efficacy of appetite modulation therapy (Lavin, Wittert et al. 1996, Flint, Gregersen et al. 2007).

Ghrelin and the GHSR-1a have also been the subject of investigation in the modulation of pain transmission (Ferrini, Salio et al. 2009). Originally, ghrelin's role in pain sensitivity was thought to be through a combination of central and peripheral GHSR-1a signalling (Guneli, Kazikdas et al. 2007, Vergnano, Ferrini et al. 2008). Chronic peripheral ghrelin administration has been shown to attenuate neuropathic pain in rats (Guneli, Onal et al. 2010). Ghrelin treatment resulted in elevated levels of anti-inflammatory cytokines *in vivo* in a rodent model of inflammatory pain (Azizzadeh, Mahmoodi et al. 2016). It has also been shown that central and peripheral ghrelin administration prevents the pain response caused by intraplantar insults (Sibilia, Lattuada et al. 2006). Furthermore, mRNA for GHSR-1a is found in pain-processing centres including the sensory motor cortex and the dorsal horn of the spinal cord (Guan, Yu et al. 1997, Hou, Miao et al. 2006, Vergnano, Ferrini et al. 2008, Zhou, Li et al. 2014). Current opinion seems to agree that ghrelin's analgesic effect is conveyed mainly through central mechanisms via the opioid system (Sibilia, Lattuada et al. 2006, Ferrini, Salio et al. 2009, Sibilia, Pagani et al. 2012, Wei, Zhi et al. 2013). Therefore, ghrelin and the GHSR-1a may have communicating peripheral and central pathways in the modulation of pain sensitivity, which may have interesting potential in the pharmacotherapy of pain.

# The challenge of changing food intake, from in vitro to in vivo

## 1.5 GHSR1a as a promiscuous target

Further to the distribution of GHSR-1a and the consideration of central and peripheral effects, the receptor is known to display heterogenous signalling cascades, downregulation/internalization and heterodimerization—all of which are akin to other GPCR's and constitute important considerations for appetite modulation therapy (Luttrell 2008) (Figure 1.4). Downstream effects of the GHSR-1a via coupling to different G-proteins have been reviewed in detail elsewhere (Schellekens, Dinan et al. 2013). Importantly, it is worth emphasising that the GHSR-1a displays heterogenous functions dependant on the location of the receptor expression in the body. For example, in neurons of the arcuate nucleus, ghrelin acting on the GHSR-1a induces orexigenic neuropeptide Y release through N-type voltage-gated $Ca_{2\beta}$ channels via cyclic adenosine monophosphate (cAMP) increases in the cell (Kohno, Gao et al. 2003). In pituitary cells responsible for effecting somatotrophin release, GHSR-1a mainly acts via $G_{\alpha q}$ coupled G-protein to trigger calcium release from intracellular stores (Malagon, Luque et al. 2003). These signalling pathways are both excitatory-interestingly, in the periphery, ghrelin binding to GHSR-1a in pancreatic β cells leads to an inhibition of cAMP and hyperpolarization of the cell (Dezaki, Kakei et al. 2007).

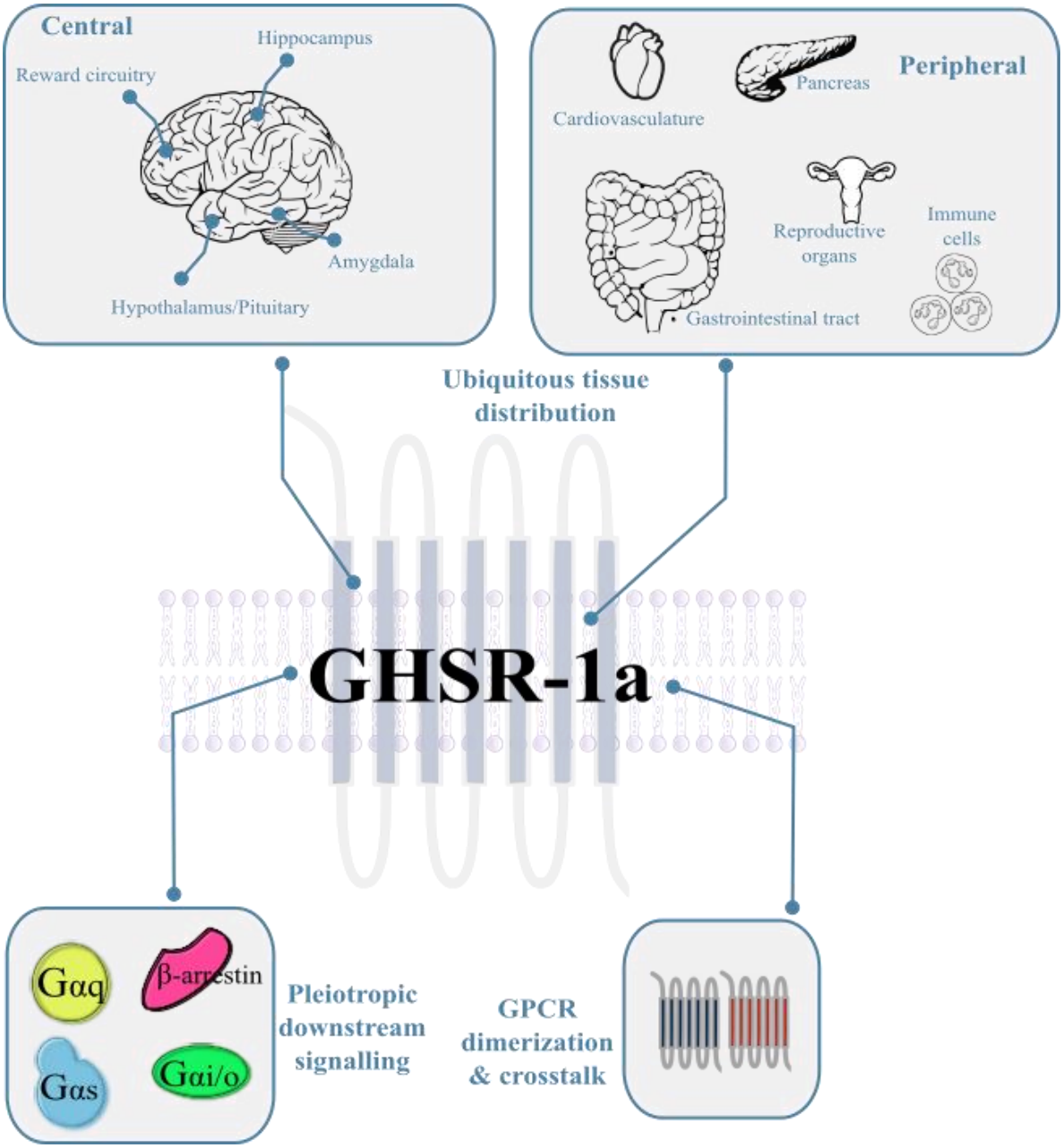



***Figure 1.4. Ubiquitous distribution and pleiotropic pharmacodynamics of GHSR-1a:*** *The GHSR-1 displays heterogenous tissue distribution as well as signalling cascades. The tissue distribution of GHSR-1a spans various areas of the brain involved in appetite, reward, memory, fear amongst others. Peripheral GHSR-1a is also widely distributed. Furthermore, GHSR-1a displays pleiotropic downstream signalling cascades as well as ability to heterodimerize and cross-talk with other GPCR systems. The widespread tissue distribution as well as the wide-ranging nature of GHSR-1a signalling leads to various downstream pharmacodynamic effects and differing behavioural outcomes.*

The GHSR-1a not only exhibits site- and ligand-dependant signalling; it demonstrates an ability to "cross-talk" with other neuroendocrine GPCRs (Schellekens, van Oeffelen et al. 2013) (Figure 1.4). The receptor has been shown to pair or dimerize with other receptors, leading to either attenuation or augmentation of signalling. GHSR-1a: melanocortin-3 receptor protomers have been described; melanocortin-3 receptor is an important downstream signalling receptor in the homeostatic control of food intake (Irani, Xiang et al. 2011). Rediger and colleagues showed that the signalling modalities of one GPCR was dependent on the conformational activity of the other. In essence, ghrelin-induced GHSR-1a activation is attenuated by interaction with the melanocortin-3 receptor (Rediger, Piechowski et al. 2011). We previously demonstrated the existence of GHSR-1a: Serotonin 2C dimers *in vitro*, hypothesizing novel pharmacological targets for drug treatment based on the involvement of serotonin 2C receptor in satiety signalling (Miller 2005, Dutton and Barnes 2006, Garfield and Heisler 2009, Schellekens, van Oeffelen et al. 2013). Furthermore, GHSR-1a: Dopamine D2 receptor co-expressed on neurons leads to attenuated dopaminergic response upon administration of a GHSR-1a antagonist *in vivo* (Kern, Albarran-Zeckler et al. 2012). Critically, it is the allosteric interaction of the GPCR protomer which results in the observed cross-talk, rather than the net effect of independent neuroendocrine signalling (Kern, Albarran-Zeckler et al. 2012). More recently, it was shown that hippocampal-dependent synaptic plasticity is modulated by GHSR-1a: Dopamine D1 heterodimerization (Kern, Mavrikaki et al. 2015). Moreover, an inactive isoform of GHSR-1a, the GHS-R1b, is worthy of mention here though it is not a major focus of this work. GHSR-1b is a truncated, 5-transmembrane receptor (Chow, Sun et al. 2012). The GHSR-1b receptor exhibits widespread tissue distribution and exhibits an ability to co-localize with the GHSR-1a causing a subsequent attenuation of activity through an increased internalization of the active receptor. This is potentially significant in the backdrop of ghrelin signalling as the GHSR-1a exhibits high constitutive signalling in the absence of its native ligand (Holst and Schwartz 2004, Petersen, Woldbye et al. 2009, Mear, Enjalbert et al. 2013).

As well as heterogenous signalling and neuroendocrine cross-talk, the expression of the GHSR-1a on the cell membrane is critical to it being a successful therapeutic target. However, GPCRs are known to downregulate via receptor

internalization or endocytosis causing a subsequent attenuation of effect (Tsao and von Zastrow 2000). Unsurprisingly, the GHSR-1a has been shown to downregulate in response to various stimuli, including ghrelin- and ghrelin-ligand mediated activation (Kaji, Kishimoto et al. 2001, Orkin, New et al. 2003, Camina, Carreira et al. 2004). After binding of ghrelin to GHSR-1a, the complex is internalised into clathrin-coated pits, from which the receptor needs to be recycled back to the surface of the cell (Orkin, New et al. 2003). *In vitro* GH release is rapidly desensitized after exposure to a ghrelin agonist, MK-0677, and *in vivo* response in beagles was reduced to 25% after 4 days of daily administration (Guyda 2002). In line with this, GH release declines rapidly upon repeated ghrelin administration in humans (Gardiner and Bloom 2008). There is a dearth of information in the literature to suggest an ability of ghrelin to sustain elevated food intake in animals or humans upon long-term administration, and it is feasible that downregulation would contribute to a decline in orexigenic effects over time. One study showed no overall effect on food intake in rats after chronic administration of acyl-ghrelin (Davies, Kotokorpi et al. 2009). A limited number of clinical studies have failed to show an appreciable difference in food intake with chronic administration of ghrelin (Lundholm, Gunnebo et al. 2010) or the synthetic agonist growth hormone releasing peptide-2 (Mericq, Cassorla et al. 2003). However, in acute situations consistently pronounced orexigenic effects are reported in both animals and humans (Wren, Seal et al. 2001, Druce, Wren et al. 2005, Schmid, Held et al. 2005, Neary, Druce et al. 2006). Conversely, GHSR-1a has been shown to upregulate, in the hypothalamus at least, during fasting (Petersen, Woldbye et al. 2009). Hence, GHSR-1a expression levels, and subsequent effect of receptor modulation, are heavily dependent on the metabolic state. To further confirm this, it has been noted that leptin-deficient Zucker rats, characterized by profound hyperphagia, display a heightened expression of the GHSR-1a and a corresponding increased sensitivity to ghrelin and ghrelin agonists (Hewson, Tung et al. 2002).

In summary, the above described heterogeneity of the GHSR-1a in terms of distribution, downstream signalling, tachyphylaxis and neuroendocrine communication paints a complex picture. This complexity has hindered development of an effective GHSR-1a targeting therapy for appetite modulation. It seems that the effect of GHSR-1a modulation hinges on the metabolic backdrop in which the therapy

is delivered, hence the indiscriminate targeting of the GHSR-1a with non-specific systemic delivery of varying ligands may be one of the reasons for a lack of efficacy to date. The widespread nature of the receptor in the body leads to GHSR-1a activation in off-target sites, potentially leading to local effects which can ultimately inhibit the intended benefit.

## 1.6 Ghrelin and Ghrelin Ligands: Pharmacokinetic Perspectives

On the whole, central action seems to be critical for GHSR-1a-mediated appetite modulation and energy balance. Understanding the pathway by which peripheral ghrelin acts centrally, after either endogenous release or exogenous administration, is critical to achieving therapeutic exploitation. As mentioned earlier, the question of whether ghrelin peptide is expressed in the brain is controversial and the subject of debate. Ghrelin immuno-reactive cells have been reported in the hypothalamus in some studies (Lu, Guan et al. 2002, Cowley, Smith et al. 2003), while the existence of ghrelin-producing cells was reported in the Arc of the hypothalamus (Kageyama, Kitamura et al. 2008). Recent evidence seems to refute these claims and now it is thought ghrelin is only present in these areas due to access of circulating ghrelin from the periphery (Furness, Hunne et al. 2011, Cabral, De Francesco et al. 2015, Perello and Dickson 2015). The main pathways by which ghrelin is thought to exert its orexigenic effect after it is released from the stomach have been extensively reviewed (Horvath, Castaneda et al. 2003).

### 1.6.1 Blood Brain Barrier Penetration

The orexigenic effects of ghrelin have immediate onset, with food intake increasing 10 min after systemic administration (Cummings, Purnell et al. 2001, Cabral, De Francesco et al. 2015). It follows therefore that ghrelin must have ready access into the brain. In fact, ghrelin can directly cross the blood brain barrier (BBB) at areas which are not highly protected, and subsequently convey its effect via neural projections from the site of entry to various feeding centres (Inui 2001, Andrews 2011). This is supported by the suggested “leaky” nature of the BBB surrounding the circumventricular organs of the brain (Banks 2002, Wang, Saint-Pierre et al. 2002, Takayama, Johno et al. 2007). The fenestrated endothelia surrounding the hypothalamus are supplied by capillaries which confer a rich blood supply, allowing the hypothalamus to sample the contents of the systemic circulation (Ciofi, Garret et al. 2009). This affords many central nervous system (CNS) active peptides, including ghrelin, access to the CNS while still retaining effective and selective barrier function for the brain (Zigman, Jones et al. 2006, Cabral, Valdivia et al. 2014). Furthermore,

the blood—cerebrospinal fluid (CSF) barrier which exists at the choroid plexus also has been shown to allow ghrelin access to the Arc. This is composed of a differentiated layer of cells that surround a core of capillaries in some brain ventricles and produce CSF, and/or the hypothalamic tanycytes, a specialized layer of bipolar ependymal cells that line the floor of the third ventricle and bridge the CSF and the capillaries of the median eminence (Redzic, Preston et al. 2005, Bolborea and Dale 2013). Other circumventricular organs such as the area postrema, a part of the dorsal vagal complex, affords ghrelin diffusive access to the abundance of GHSR-1a's in the nucleus tractus solitarius and dorsovagal nucleus. The nucleus tractus solitarius (NTS) is a relay hub for appetite regulation with a complex network of efferent and afferent connections. The NTS converts humoral responses into neuronal communication (Grill and Hayes 2012).

### 1.6.2 Vagus Nerve Signalling

The NTS is also important to the other described route by which peripheral ghrelin accesses central GHSR-1a; remote modulation from the gut signalling through the vagus nerve and the brainstem (Date, Murakami et al. 2002, Horvath, Castaneda et al. 2003). Indeed, several gastrointestinal hormones such as cholecystokinin (CCK), peptide YY, and glucagon-like peptide 1 (GLP-1), transmit orexigenic and satiating signals to the brain, at least in part, via vagal afferents (Smith, Jerome et al. 1981, Koda, Date et al. 2005, Abbott, Small et al. 2006). Feeding-related information can travel directly to the dorsal vagal complex and NTS, where signals are converted from humoral to neural format and further relayed to higher brain levels. Indeed, it is known that gut derived peptides such as the satiating CCK exert their central action via vagal afferents from the gastrointestinal tract (Rogers and Hermann 2008). Early studies using c-Fos expression as a marker of neuronal activation showed that peripheral administration of a ghrelin mimetic increased Fos protein in the NTS (Bailey, Smith et al. 1998). The NTS provides a direct noradrenergic projection to the hypothalamus which is believed to be important for neural regulation of energy balance and food intake (Smith, Sun et al. 2007). Date and colleagues demonstrate that peripheral ghrelin signalling reaches the NTS by either blood or neural mechanisms and relays noradrenergic stimuli to the hypothalamus to increase feeding (Date, Murakami et al.

2002, Date, Shimbara et al. 2006, Date 2012). Transections above the level of the NTS, or specific ablation of dopamine β-hydroxylase (the noradrenaline synthesizing enzyme), abolished peripheral ghrelin-induced feeding (Date, Shimbara et al. 2006). Moreover, it has been reported that the orexigenic action of ghrelin is attenuated in humans who underwent gastric surgery involving complete or partial vagotomies (le Roux, Neary et al. 2005). Vagotomy also abolishes the orexigenic activity of ghrelin in rats (Date, Murakami et al. 2002). Another preclinical study however, reports that ghrelin's orexigenic effect remains intact after a sub diaphragmatic vagal deafferentiation. The authors argue that a bilateral vagotomy, as described in Date's work, would indiscriminately remove both afferent and efferent vagal innervation, thereby severing a multitude of other physiological processes, including satiating signals (Arnold, Mura et al. 2006). It is thus stated that sub diaphragmatic vagal deafferentiation is a more representative model for ablating the vagal afferent connection as it is less invasive to other vagally-mediated physiological parameters such as heart rate and respiration. However, the dose of ghrelin used in this study was substantially higher than that used in the original work by Date therefore results cannot be directly compared. Critically, it points to the fact that vagal signalling is not essential to relay ascending orexigenic messages, likely due to the fact that the area postrema can facilitate diffusive access of ghrelin from the bloodstream to the NTS, enabling ascending signalling even without vagal innervation of the NTS. This is supported by the fact that intravenous ghrelin administration stimulates GH secretion in vagotomised patients (Takeno, Okimura et al. 2004). Taken together, all of the above information strongly suggests an interlinked role between blood and neural pathways for conveying ghrelin's signal from the periphery to the CNS.

## 1.7 Ghrelin administration in Human Studies

Normal serum ghrelin levels vary in man and reach 0.2–0.4 pmol/mL in hunger states (Akamizu, Takaya et al. 2004, Druce, Wren et al. 2005), with active ghrelin levels peaking at of 0.01–0.035 pmol/mL (Akamizu, Iwakura et al. 2008, Paulo, Brundage et al. 2008, Veldhuis, Reynolds et al. 2008, Ashby, Ford et al. 2009). Intravenous infusions of 1–40 pmol/kg/min active ghrelin have been used clinically to increase appetite acutely in cachectic states (Wren, Seal et al. 2001, Rigamonti,

Pincelli et al. 2002, Druce, Wren et al. 2005, Levin, Edholm et al. 2006, Strasser, Lutz et al. 2008). From a pharmacological perspective, doses in this range are supraphysiological and have resulted in several hundred-fold changes in both active and total plasma ghrelin (Table 1.1). Lippl and colleagues administered doses of ghrelin more representative of the levels experienced endogenously, resulting in active ghrelin increasing to 0.057 pmol/mL (2.4-fold increase from baseline) (Lippl, Erdmann et al. 2012). This elevation failed to show an orexigenic effect in participants (Lippl, Erdmann et al. 2012). Critically, endogenous active ghrelin reaches similar levels after overnight fasting (0.1–0.35 pmol/mL) (Akamizu, Takaya et al. 2004, Druce, Wren et al. 2005, Tong, Dave et al. 2013), predictably stimulating food intake and increasing incentive salience of food (Cowley, Smith et al. 2003, Druce, Wren et al. 2005). However, higher levels of plasma active ghrelin (>1.6 pmol/mL) have been required to produce an appetite-stimulating effect in clinical studies (Druce, Wren et al. 2005). This may be indicative of the fact that many studies administer ghrelin in fasted states, therefore necessitating a higher dose in order to overcome elevated basal ghrelin levels. Indeed, Lippl and colleagues was the only study which administered ghrelin in the fed state to patients, and therefore had low basal levels of ghrelin (Table 1.1). It also may be a reflection that many studies fail to account for des-acyl ghrelin. This was originally thought to be a pharmacologically inactive breakdown product of active ghrelin but recent evidence has shown this is not the case (Delhanty, Neggers et al. 2014)

***Table 1.1 Summary of clinical dosing studies involving ghrelin:*** *The acylation status, dose, food intake status and form of ghrelin assayed are summarised in the below table for the various clinical studies involving ghrelin.*

| **Status** | **Dose of Infusion (Duration)** | **Fed Status** | **Form Assayed** | **Mean Serum Ghrelin (pmol/mL)** | **Average Fold Increase** | **Time Post-Dose (min)** | **Outcome of Study** | **Reference** |
|---|---|---|---|---|---|---|---|---|
| Acylated | 5 pmol/kg/min (180 min) | Overnight fasted | Total | 1.32 | Not reported | 180 ($T_{max}$) | Described a 2-compartment model of ghrelin kinetics | (Vestergaard, Hansen et al. 2007) |
| Acylated<br>Acylated | 300 pmol/kg (Bolus)<br>1500 pmol/kg (Bolus) | Overnight fasted | Total and active | Total: 1.06<br>Acylated: 0.447<br>Total: 6.598<br>Acylated: 3.454 | 4.58<br>18.7<br>28.6<br>145.1 | 15 ($T_{max}$)<br>15 ($T_{max}$) | Established clinical safety of ghrelin for disorders of appetite | (Akamizu, Takaya et al. 2004) |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Acylated | 3000 pmol/kg (Bolus) | Overnight fasted | Total | 44.5 | 61 | 1 | Plasma ghrelin shown to be elevated in cachexia | (Nagaya, Kojima et al. 2001) |
| Acylated | 5 pmol/kg/min (65 min)<br>15 pmol/kg/min (65 min)<br>25 pmol/kg/min (65 min) | Overnight fasted | Total and active | Total: 1.647<br>Acylated: 1.170<br>Total: 5.139<br>Acylated: 3.510<br>Total: 8.619<br>Acylated: 5.880 | Not reported<br>118<br>Not reported<br>355<br>Not reported<br>594 | 45 ($T_{max}$) | Acyl- and desacyl ghrelin have different metabolism. Acyl-ghrelin can deacylate readily. | (Tong, Dave et al. 2013) |
| Acylated | 84 pmol/kg (Bolus) + 5 pmol/kg/min (65 min) | Overnight fasted | Active and inactive | Acylated: 0.579<br>Desacylated: 0.350 | 44<br>17 | 30 ($T_{max}$) | As above | (Tong, Dave et al. 2013) |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Desacylated | 343 pmol/kg + 20.8 pmol/kg/min (65 min) | Overnight fasted | Active and inactive | Acylated: 0.006<br>Desacylated: 4.955 | No change<br>233 | Not specified | As above | (Tong, Dave et al. 2013) |
| Acylated and Desacylated | Acylated:<br>84 pmol/kg (Bolus) + 5 pmol/kg/min (65 min)<br>Desacylated:<br>343 pmol/kg + 20.8 pmol/kg/min (65 min) | Overnight fasted | Active and inactive | Acylated: 0.495<br>Desacylated: 4.644 | 54<br>272 | Not specified | As above | (Tong, Dave et al. 2013) |
| Acylated | 1 pmol/kg/min (75 min)<br>5 pmol/kg/min (75 min) | Overnight fasted | Total | 0.725<br>1.598 | 1.6<br>3.6 | 45 ($T_{max}$)<br>45 ($T_{max}$) | Ghrelin increases food intake and lean and obese subjects | (Druce, Wren et al. 2005) |
| Acylated | 1 pmol/kg/min (120 min)<br>5 pmol/kg/min (120 min) | Not specified | Total | 0.958<br>4.087 | 3.54<br>15.13 | 90<br>90 | Ghrelin fails to stimulate food intake in | (le Roux, Neary et al. 2005) |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | | vagotomised patients | |
| Acylated | 0.3 pmol/kg/min (300 min) | Fed | Active | 0.057 | 2.4 | 210 ($T_{max}$) | Low-dose ghelin infusion fails to increase food intake | (Lippl, Erdmann et al. 2012) |
| Acylated | 7.5 pmol/kg/min (120 min)<br>15 pmol/kg/min (120 min) | Overnight fasted | Total | 0.300<br>0.494 | 2<br>3 | 120 ($T_{max}$)<br>120 ($T_{max}$) | Large doses of ghrelin increase several pituitary and adrenal hormones | (Lucidi, Murdolo et al.) |
| Acylated | 3600 pmol/kg (Subcutaneous) | Overnight fasted | Total and active | Total: 0.988<br>Acylated: 0.355 | 5.15<br>10.23 | 15 ($T_{max}$)<br>30 ($T_{max}$) | Acyl-ghrelin but not des-acyl increases food intake | (Druce, Neary et al. 2006) |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | | in fed and fasted state | |
| Acylated | 300 pmol/kg (Subcutaneous)<br>1500 pmol/kg<br>3000 pmol/kg | Overnight fasted | Total | ~ 0.350<br>~ 0.900<br>~ 1.400 | 2<br>8<br>12 | 30 ($T_{max}$) | Left ventricular ejection fraction increased by ghrelin | (Enomoto, Nagaya et al. 2003) |

### 1.7.1 Acyl and Desacyl- Ghrelin—Implications for Therapeutic Approaches

Both acylated and des-acylated forms of the hormone ghrelin are detected in the peripheral circulation (Delhanty, Neggers et al. 2012). Despite this, many studies assessing endogenous ghrelin levels in blood fail to specify the acylation status of the hormone (Stark, Santos et al. 2016). In fact, only some preclinical studies have distinguished between the effects of acyl- and desacyl-ghrelin (Andrews, Erion et al. 2009, Bayliss and Andrews 2013, Bayliss, Lemus et al. 2016). Furthermore, it is critical for accurate measurement of acyl- ghrelin that blood samples are appropriately stabilized in order to prevent des-acylation (Liu, Prudom et al. 2008, Delhanty, Neggers et al. 2014). The binding of acyl-ghrelin and subsequent activation of GHSR-1a is well established (Kojima, Hosoda et al. 1999, Bednarek, Feighner et al. 2000). Similarly, the lack of desacyl- ghrelin binding to GHSR-1a is described (Kojima, Hosoda et al. 1999). Desacyl-ghrelin does not compete with acyl-ghrelin for GHSR-1a binding at physiological concentrations (Veldhuis and Bowers 2010), however, it has been shown to activate the receptor at supraphysiological concentrations (Gauna, Van de Zande et al. 2007, Heppner, Piechowski et al. 2014). Desacyl-ghrelin is the most abundant form in the circulation and is purported to be the active ligand for additional, as yet unknown, GHSR subtypes (Broglio, Gottero et al. 2004, Schellekens, Dinan et al. 2013, Delhanty, Neggers et al. 2014).

Peripheral acyl-ghrelin administration markedly increases circulating GH, prolactin, adrenocorticotrophic hormone, and cortisol levels (Broglio, Gottero et al. 2004). This is accompanied by a decrease in insulin and a concomitant increase in plasma glucose. Interestingly, although desacyl-ghrelin administration had no such effects in isolation, when administered in combination with acyl-ghrelin it was able to negate the observed effects on plasma insulin and glucose (Broglio, Gottero et al. 2004). Indeed, it has been suggested that desacyl-ghrelin should be considered as a hormone distinct from acyl-ghrelin given its ability to elicit effects on certain peripheral actions such as cardiovasculature, cell proliferation and certain aspects of adiposity (Broglio, Gottero et al. 2004). Overnight intravenous desacyl-ghrelin infusion was found to improve glucose metabolism and, conversely to acyl-ghrelin, display a glucose-lowering effect (Benso, St-Pierre et al. 2012). Moreover, combined administration of acyl- and desacyl-ghrelin strongly improved insulin sensitivity compared to acyl-ghrelin administration alone (Gauna, Meyler et al. 2004). Therefore, desacyl-ghrelin can

be metabolically active in an opposing manner to acyl-ghrelin to improve glycemic control. Furthermore, *in vivo* work has shown that desacyl-ghrelin alone does not alter food intake, but in keeping with the observed metabolic effects, attenuates acyl-ghrelin -induced food intake and arcuate nucleus neuronal activation (Neary, Druce et al. 2006, Inhoff, Monnikes et al. 2008, Kumar, Salehi et al. 2010). It has also been suggested that desacyl-ghrelin acts independently of acyl-ghrelin via the hypothalamus to decrease food intake and gastric motility (Asakawa, Inui et al. 2005), and central desacyl-ghrelin administration was reported to increase food intake via activation of orexin neurons in the LH (Toshinai, Yamaguchi et al. 2006). It has been further demonstrated that intracerebroventricular and intravenous injections of desacyl-ghrelin disrupted fasted motor activity in the stomach (Chen, Inui et al. 2005). For further information the reader is directed towards a comprehensive review by Soares and colleagues which summarise effects of both isoforms on the various systems and organs (Soares and Leite-Moreira 2008).

The pharmacokinetic parameters of infused acyl-ghrelin, desacyl-ghrelin, or a combination thereof in healthy subjects have been reported. The plasma half-life of acyl-ghrelin was 9–11 min after an intravenous infusion, whereas the half-life of total ghrelin (acyl-ghrelin + desacyl-ghrelin) was 35 min, indicating that desacyl-ghrelin has a slower clearance than acyl-ghrelin (Tong, Dave et al. 2013). Similar estimates of half-lives have been reported elsewhere (Akamizu, Takaya et al. 2004, Paulo, Brundage et al. 2008). It is estimated that the ratio of des-acylated: acylated form of ghrelin in the plasma exceeds 9:1 (Hosoda, Kojima et al. 2003, Bang, Soule et al. 2007, Takagi, Legrand et al. 2013). However, during an infusion of acyl-ghrelin, the ratio of desacylated: acylated is 2:1. Interestingly, it was also shown that acyl-ghrelin infusion is responsible for an absolute increase in circulating plasma levels of desacyl-ghrelin (Tong, Dave et al. 2013). This indicates that upon entry to the circulation, acyl-ghrelin is de-acylated, hence leading to an increase in desacyl-ghrelin, which potentially counters the effects of acyl-ghrelin. Interestingly, in Prader-Willi syndrome, patients with an elevated ratio of acyl- to desacyl-ghrelin show pronounced hyperphagia and weight gain compared to those patients who display a normal acyl:desacyl ratio (Kuppens, Diene et al. 2015). Therefore, acyl-ghrelin and desacyl-ghrelin not only exhibit different clearance rates from the circulation, but acyl-ghrelin is de-acylated in plasma. It is estimated that acyl-ghrelin accounts for only half of the increase in total ghrelin levels after dosing of acyl-ghrelin (Akamizu, Takaya et al. 2004). In this respect, active de-acylating enzymes have been

identified in the circulation (Satou, Nishi et al. 2010). The ratio of desacyl- ghrelin: acyl-ghrelin can also change pending the metabolic state i.e. hunger can increase circulating acyl-ghrelin (Liu, Prudom et al. 2008, Kirchner, Gutierrez et al. 2009). Given the proposed opposing effects of acyl- and desacyl- ghrelin, and the variable information in the literature vis-à-vis pharmacokinetic disposition, due consideration is warranted in the interpretation of trials to date.

### 1.7.2 Synthetic Ghrelin Ligands

The short half-life of acyl-ghrelin, the ubiquitous expression of GHSR-1a and the often-overlooked presence of a functional antagonist in desacyl- ghrelin, leads to an unpredictable relationship between the pharmacokinetics and pharmacodynamics of ghrelin. Numerous synthetic ghrelin ligands have been developed over the years, all of which are more stable and exhibit a longer duration of action than native acyl-ghrelin (Moulin, Brunel et al. 2013, Vodnik, Štrukelj et al. 2016). From a pharmacokinetic perspective, increased half-life of synthetic compounds will lead to increased penetration into tissues and activation of the GHSR-1a for prolonged periods due to greater stability. In addition, synthetic derivatives are not converted to desacyl-ghrelin and avoid any potential counter effects. This therefore should lead to more predictable relationships of pharmacokinetics with pharmacodynamic effect.

Pharmacokinetic data is sparse for synthetic ligands, with many trials solely reporting on pharmacodynamic outcomes (Table 1.2). This is largely due to the focus of the field of research on ghrelin shifting over time. The first clinical studies mainly focus on ghrelin and ghrelin ligands as GH secretagogues, thus solely measuring GH response and failing to measure serum ghrelin (Arvat, Maccario et al. 2001, Broglio, Benso et al. 2003). Indeed, it must be borne in mind that ghrelin had yet to be discovered for certain studies (Deghenghi, Cananzi et al. 1994, Patchett, Nargund et al. 1995, Ghigo, Arvat et al. 1996, Pihoker, Badger et al. 1997, Hansen, Raun et al. 1999, Phung, Sasaki et al. 2001). Originally, compounds such as Growth Hormone Releasing Peptide 6 (GHRP-6) and GHRP-2 were developed as somatotrophin secretagogues with the aim of treating GH deficiency syndromes such as pituitary dwarfism (Deghenghi, Cananzi et al. 1994, Ghigo, Arvat et al. 1996, Okada, Ishii et al. 1996, Pihoker, Badger et al. 1997, Torsello, Luoni et al. 1998, Lee, Vega et al. 2000, Roumi, Marleau et al. 2000, Phung, Sasaki et al. 2001, Laferrere, Abraham et al. 2005). At the time of

ghrelin's discovery, focus shifted towards the possibility of exploiting these compounds for disorders of appetite (Lawrence, Snape et al. 2002, Cowley, Smith et al. 2003, Horvath, Castaneda et al. 2003, Inui, Asakawa et al. 2004). With the increased appreciation of the role of ghrelin, research shifted to investigate its effects on the mesolimbic reward circuitry (Kawahara, Kawahara et al. 2009, Egecioglu, Jerlhag et al. 2010, Dickson, Egecioglu et al. 2011, Skibicka, Hansson et al. 2011). More recently, ghrelin agonists have been explored as gastrointestinal prokinetics to treat idiopathic and diabetic gastroparesis, as well as post-operative ileus (Sanger 2008, Charoenthongtrakul, Giuliana et al. 2009, De Smet, Mitselos et al. 2009). Preclinical studies are thus difficult to directly compare due to variable approaches to dosing and vastly different experimental setups and outcome.

Nevertheless, the physiological mechanisms of appetite stimulation, body weight and other parameters for synthetic ligands (Table 1.2) are mediated through interaction with the GHSR-1a, and thus are broadly similar to ghrelin itself. Unfortunately, given the sparsity of comprehensive pharmacokinetic studies, many of parameters in Table 1.2 were taken from preclinical study data. No GHSR-1a antagonists or inverse agonists have been used clinically and there is a paucity of pharmacokinetic data available, hence they were not included in the scope for Table 1.2, however the reader is directed to a recent review for further information on these compounds (Vodnik, Štrukelj et al. 2016). Additionally, it is unwise to utilise pharmacodynamic outcomes as a surrogate measurement to compare ligand efficacy, due to heterogenous receptor-ligand interaction as discussed above (Sivertsen, Lang et al. 2011). For example, GH output is poorly correlated with orexigenic effect or body weight gain *in vivo*—stimulation of GH without affecting food intake has been demonstrated (Torsello, Luoni et al. 1998). The agonist ulimorelin fails to elicit any GH release after both central and peripheral administration (Hoveyda, Marsault et al. 2011). Anamorelin displays three times the potency of endogenous ghrelin in activating the ghrelin receptor *in vitro* (Pietra, Takeda et al. 2014). However, it is noted this greater potency does not translate to greater *in vivo* levels of GH response (Pietra, Takeda et al. 2014). Even minimal structural modifications of GH releasing peptide analogues affect the behavioural (food intake) but not GH-releasing properties of the analogue (Torsello, Luoni et al. 1998). Paradoxically, there have even been a number of reported GHSR-1a antagonists, which display orexigenic effects. Although the antagonist BIM-28163 blocks ghrelin-induced GHSR-1a activation, and prevents GH secretion *in vivo* as a result, the compound elicits increases in food intake and body weight. However, this is

thought to be potentially due to action at a receptor other than the GHSR-1a (Halem, Taylor et al. 2005, Hassouna, Labarthe et al. 2013). Furthermore, GSK1614343 also increased food intake and body weight *in vivo*, but knockout of the GHSR-1a abolished this effect, confirming that the antagonist was working via this receptor (Costantini, Vicentini et al. 2011). Antagonists with agonistic properties *in vivo* may be explained by biased agonism (M'Kadmi, Leyris et al. 2015). Vodnik and colleagues review several ligands which display biased agonism (Vodnik, Štrukelj et al. 2016). Individual drug-receptor interactions therefore determine distinct pharmacodynamic outcomes (Moulin, Ryan et al. 2007, Depoortere 2009). Different ligands can activate signalling cascades which may be more desirable and have the potential to be exploited for the development of more selective therapeutics (M'Kadmi, Leyris et al. 2015). This has led to examination of ligands, including inverse agonists, with selective effects for certain outputs. For example agonists for treating osteoporosis through GH secretion may have the adverse effect of increasing body weight (M'Kadmi, Leyris et al. 2015). Antagonists for GHSR-1a may be developed with the ability to decrease centrally-mediated food intake and adiposity, without inhibiting GH secretion. The potential of utilising biased agonism to achieve improved therapeutic efficacy warrants further investigation and has been highlighted in recent literature (Mende, Hundahl et al. 2018, Ramirez, van Oeffelen et al. 2018).

***Table 1.2. Ghrelin agonists used clinically***. *The half-life, oral bioavailability and centrally-mediated effects have been summarised. To date, no GHSR-1a antagonists have reached clinical trials.*

| **Agonist** | **Class of Compound** | **Oral Bioavailability (Species)** | **Half Life** | **Centrally Regulated Parameters Reported** |
|---|---|---|---|---|
| Growth Hormone Releasing Peptide 6 (GHRP-6) | Synthetic peptide | 0.3% (Human) (Walker, Codd et al. 1990, Moulin, Brunel et al. 2013) | 0.3 h (Moulin, Brunel et al. 2013) | ↑ Food intake (Lawrence, Snape et al. 2002), ↑ Body weight (Bowers, Momany et al. 1984, Lawrence, Snape et al. 2002), ↑ Gastric emptying (Kitazawa, De Smet et al. 2005), ↑ Growth hormone (Bowers, Momany et al. 1984, Deghenghi, Cananzi et al. 1994) |
| Hexarelin | Synthetic peptide | <0.3% (Human) (Ghigo, Arvat et al. 1994) | 1.15 h (Ghigo, Arvat et al. 1994, Roumi, Marleau et al. 2000) | ↑ Food intake (Torsello, Luoni et al. 1998), ↑ Growth velocity (Imbimbo, Mant et al. 1994, Laron, Frenkel et al. 1995, Arvat, di Vito et al. 1997) |
| Pralmorelin (GHRP-2) | Synthetic peptide | Not reported, but has been dosed | 0.52 h (Pihoker, Kearns et al. 1998) | ↑ Food intake (Mericq, Cassorla et al. 2003, Laferrere, Abraham et al. 2005), |

| | | | | |
|---|---|---|---|---|
| | | orally (Bowers 1993) | | ↑ Growth hormone (Bowers 1993, Pihoker, Kearns et al. 1998) |
| Alexamorelin | Synthetic peptide | Not reported | Not reported | ↑ Growth hormone (Broglio, Benso et al. 2000) |
| Ipamorelin | Synthetic peptide | 1%–6% (Rat, Dog) (Ankersen, Johansen et al. 1998) | 2 h (Gobburu, Agerso et al. 1999) | ↑ Growth hormone (Ankersen, Johansen et al. 1998, Johansen, Nowak et al. 1999), ↑ Body weight (Ankersen, Johansen et al. 1998), ↑ Gastointestinal motility (Polvino, Nelson et al. 2011) |
| Capromorelin | Small molecule | 65% (Carpino, Lefker et al. 2003) (Rat) (Khojasteh-Bakht, O'Donnell J et al. 2005) | 2.4 h (Carpino, Lefker et al. 2003) | ↑ Growth hormone (Smith, Pong et al. 1996, Carpino, Lefker et al. 2003), ↑ Body weight (Pan, Carpino et al. 2001), ↑ Gastric emptying (Kitazawa, De Smet et al. 2005) |
| Relamorelin | Synthetic peptide | Not reported | 19.4 h (Lembo, Camilleri et al. 2016) | ↑ Growth hormone (Palus, Schur et al. 2011), ↑ Food intake, ↑ Body weight (Strassburg, Anker et al. 2008, Palus, von Haehling et al. 2013, Fischer, Finan et al. 2014), |

| | | | | |
|---|---|---|---|---|
| | | | | ↑ Gastric emptying (Shin, Camilleri et al. 2013, Van der Ploeg, Laken et al. 2014) |
| Macimorelin | Small molecule | Not reported, but has been dosed orally (Ali and Garcia , Garcia, Swerdloff et al. 2013) | 3.8 h (Piccoli, Degen et al. 2007) | ↑ Growth hormone (Broglio, Boutignon et al. 2002, Garcia, Swerdloff et al. 2013) |
| Tabimorelin | Synthetic peptide | 30%–35% (Rat) (Hansen, Raun et al. 1999, Ankersen, Kramer Nielsen et al. 2000) | 20.8 h (Zdravkovic, Søgaard et al. 2000, Zdravkovic, Christiansen et al. 2001) | ↑ Growth hormone (Ankersen, Kramer Nielsen et al. 2000, Zdravkovic, Søgaard et al. 2000, Zdravkovic, Christiansen et al. 2001) ↑ Body weight (Hansen, Raun et al. 1999) |
| Anamorelin | Small molecule | Not reported, but has been dosed orally (Garcia, Boccia et al. 2007, Garcia and Polvino 2007, Garcia and Polvino 2009) | 7 h (Garcia and Polvino 2009) | ↑ Growth hormone (Garcia and Polvino 2009, Garcia, Friend et al. 2013), ↑ Food intake (Garcia, Boccia et al. 2007, Garcia and Polvino 2007, Garcia, Friend et al. 2013, Pietra, Takeda et al. 2014) |

| | | | | |
|---|---|---|---|---|
| Ibutamoren (MK-0677) | Small molecule | >60% (Dog) (Patchett, Nargund et al. 1995, Svensson, Lonn et al. 1998, Adunsky, Chandler et al. 2011) | 6 h (Guyda 2002) | ↑ Growth hormone (Patchett, Nargund et al. 1995, Jacks, Smith et al. 1996, Adunsky, Chandler et al. 2011), ↑ Body weight (Prahalada, Block et al. 1999), ↑ Fat free mass (Svensson, Lonn et al. 1998) |
| Ulimorelin | Synthetic peptide | 24% (Rat) (Hoveyda, Marsault et al. 2011) | 1.6 h (Venkova, Fraser et al. 2007, Fraser, Hoveyda et al. 2008, Fraser, Venkova et al. 2009) | ↑ Growth hormone (no effect), ↑ Food intake, ↑ Gastrointestinal motility (Fraser, Hoveyda et al. 2008, Lasseter, Shaughnessy et al. 2008, Fraser, Venkova et al. 2009, Ejskjaer, Dimcevski et al. 2010, Hoveyda, Marsault et al. 2011, Wo, Ejskjaer et al. 2011) |

### 1.7.1 Clinical Status of synthetic Ghrelin Ligands

An increasing number of GHSR-1a ligands are likely to be seen in the clinic in the near future. Interestingly, anamorelin was recently refused FDA and EMA approval for the treatment of cachexia associated with non-small cell lung cancer (Garcia 2017). This has led to some debate surrounding the appropriate clinical endpoints for establishing treatment efficacy as well as calls for a greater understanding of regulators of appetite. Much of this area is uncharted regulatory territory and as such the lack of precedent for FDA/EMA guidelines serves as an impediment to selecting and powering for primary outcomes. Currently however, anamorelin is being reassessed for different primary outcomes related to anorexia-cachexia compared to the original ROMANA study, and may well attain regulatory approval in the future based on this (NCI 2018). Recently, Capromorelin has also been FDA-approved for veterinary use to stimulate appetite in dogs (Rhodes, Zollers et al. 2017). Other GHSR-1a agonists are in the pipeline, albeit not solely for the indication of appetite modulation. The synthetic agonist macimorelin is in the process of gaining regulatory approval for the diagnosis of adult GH deficiency (Garcia, Biller et al. 2018). Furthermore, phase 3 studies are underway for relamorelin in the treatment of diabetic gastroparesis (Allergan 2018).

## Enhancing efficacy through BBB penetration.

BBB penetration per se does not seem to be a key criterion for effecting changes to the centrally- mediated processes of appetite stimulation, growth hormone output or adipogenesis. This is probably due to a hijacking of the endogenous mechanisms of transport for ghrelin across the BBB and is in line with the literature on mechanism of CNS access of ghrelin discussed in the earlier parts of this review (Banks 2002, Cabral, De Francesco et al. 2015). Despite its non-centrally penetrant action, anamorelin is in phase 3 trials for the treatment of cancer-anorexia-cachexia syndrome (Garcia, Boccia et al. 2015, Zhang and Garcia 2015). The compound elicits an orexigenic effect pointing to a central mechanism much in line with ghrelin's homeostatic action, with a lack of traditional CNS penetration. This is also the case

for other non-centrally penetrant compounds (Torsello, Luoni et al. 1998, Laferrere, Abraham et al. 2005). Given the expression of the GHSR-1a in less accessible brain areas, particularly in relation to incentive salience, there is an impetus to investigate BBB penetrability of ghrelin ligands further.

Preclinical work has already shown the potential benefits of BBB penetrant ghrelin agonists in other therapeutic areas. Activation of GHSR-1a in the spinal cord activates colonic motility. In the rat, severing the spinal cord at a thoracic level prevented defecation induced by the centrally penetrant agonist CP464709 (Shimizu, Chang et al. 2006). Critically, this stimulation of colorectal activity was evident after *peripheral* administration of the ghrelin agonist, indicating a direct action on GHSR-1a in lumbosacral defecation centres. Furthermore, the lack of effect of peripheral ghrelin on the colon *in vivo* demonstrates the importance of BBB penetration (Trudel, Tomasetto et al. 2002). GSK 894281 is an orally bioavailable BBB-penetrant ghrelin agonist which causes a prompt and dose-related output of faecal pellets after administration (Shafton, Sanger et al. 2009). HM01 is another such agonist in preclinical trials as a colokinetic; again, its prokinetic action is attributed to its ability to cross the BBB and act on GHSR-1a's present in the nerves of the lumbar section of the spinal cord (Naitou, Mamerto et al. , Karasawa, Pietra et al. 2014, Naitou, Mamerto et al. 2015, Borner, Loi et al. 2016).

Centrally penetrant GHSR-1a antagonists reduced body weight in diet-induced obese (DIO) mice when administered for 10 days, while also improving glucose tolerance (Esler, Rudolph et al. 2007, Rudolph, Esler et al. 2007). Conversely, a non CNS-penetrating antagonist demonstrated comparatively mild effects on body weight, while retaining an effect on the peripherally regulated glucose tolerance. It has been postulated that the efficacy of these compounds on food intake and body weight appears to be correlated with their ability to antagonize central vs. peripheral GHSR-1a's in different animal models (Moran and Dailey 2009); YIL 870 and YIL 781 are quinazolinone-derived GHSR-1a antagonists which differ mainly in their ability to traverse the BBB. YIL 870 produces greater anorexigenic and weight reducing effects in diet-induced obese mice vs. the non-penetrant YIL 781, while both yielded a comparative improvement in glucose tolerance which has a peripheral element to its

regulation (Esler, Rudolph et al. 2007). Robust evidence thus shows that for antagonists to be effective in regulating body weight they need to cross the BBB. Pharmacological evaluation in obesity-induced rats revealed that a BBB penetrant inverse agonist for the GHSR-1a effectively reduced weight gain (Takahashi, Funami et al. 2015). *Ad libitum* food intake was also reduced in mice treated with a BBB-penetrant inverse agonist (AZ-GHS-38) while a lack of efficacy was obtained in mice treated with a non- BBB-penetrant inverse agonist (McCoull, Barton et al. 2014). Therefore, a crucial determinant of the anti-obesogenic potential of GHSR-1a inverse agonists and antagonists is their ability to traverse the BBB.

The effect of ghrelin antagonists on the mesolimbic dopaminergic pathway has been investigated in the context of addictive-like behaviour. JMV 2959 is a centrally active GHSR-1a antagonist found to effectively reduce rewarding properties of addictive substances (Jerlhag and Engel 2011, Skibicka, Hansson et al. 2012, Engel, Nylander et al.). Systemic administration of JMV attenuated ghrelin-induced motivation to work for sugar pellet reward (Skibicka, Hansson et al. 2012) in an operant conditioning paradigm. It was found that cocaine and amphetamine-induced place preference and extracellular accumbal DA were attenuated by administration of JMV 2959. This demonstrates a role for the GHSR-1a in the pathogenesis of addiction, while also suggesting the importance of ligand access to less accessible brain areas. These findings also generalise to opioid-induced DA release (Sustkova-Fiserova, Jerabek et al. 2014, Engel, Nylander et al. 2015). Notably, Jerlhag and colleagues have also concluded that BBB penetrant GHSR-1a antagonists may have potential in alcohol use disorders (Jerlhag, Egecioglu et al. 2009).

## 1.8 Hunger Is the Best Sauce—Targeting the Mesolimbic Reward Circuitry

The old adage that “hunger is the best sauce” may provide a potential novel approach for appetite modulation therapies - food becomes more appealing the hungrier we are (Perello and Dickson 2015). This is an evolutionally-procured mechanism for survival in order to promote food intake beyond the immediate metabolic demand, to compensate for times of food scarcity (Lenard and Berthoud 2008). The unravelling role of ghrelin and the expression of GHSR-1a in a number of

brain areas associated with reward, meant that it became implicated in food-reward directed behaviour (Egecioglu, Jerlhag et al. 2010, Skibicka, Hansson et al. 2011, Skibicka, Hansson et al. 2012). Consequently, the GHSR-1a may be a driver in the decision to eat palatable, calorie-dense foods, often beyond metabolic need. The role which ghrelin is purported to play at the interface between homeostatic and hedonic food intake regulation has been reviewed (Dickson, Egecioglu et al. 2011, Egecioglu, Skibicka et al. 2011, Schellekens, Dinan et al. 2013). We have previously summarised recent experiments examining ghrelin's effect on rewarding food intake and preference (Schellekens, Dinan et al. 2013). It is now generally accepted that food intake is the result of an integrated multi-process neuro-circuit, involving the cortex and critically, the mesolimbic dopaminergic system—therefore, targeting GHSR-1a in the midbrain reward system, with BBB-penetrant ligands, may hold novel therapeutic potential.

One of the key areas expressing the GHSR-1a in this respect is the VTA. The importance of dopaminergic VTA outputs in feeding has been well established (Wise 2006, Fields, Hjelmstad et al. 2007, Narayanan, Guarnieri et al. 2010). Central ghrelin administration recruits dopaminergic neurons in the VTA and results in an elevated dopaminergic tone in the NAcc of mice, while more targeted intra-VTA administration robustly increases the intake of both standard chow (Naleid, Grace et al. 2005, Abizaid, Liu et al. 2006) and palatable food (Skibicka, Hansson et al. 2011, Skibicka, Shirazi et al. 2013). Incidentally, ghrelin administration into the medial prefrontal cortex also induces palatable-reward seeking behaviour in rats (Parent, Amarante et al. 2015). Microdialysis and electrophysiological studies in rodents have shown that peripheral ghrelin enhances dopaminergic neuronal firing, synapse formation and DA turnover in the NAcc. In animals, peripheral ghrelin treatment has increased locomotor activity and motivation to work for food, while also shifting food preference towards calorie dense and palatable foods (Jerlhag, Egecioglu et al. 2007, Egecioglu, Jerlhag et al. 2010, Perello, Sakata et al. 2010, Dickson, Egecioglu et al. 2011, Skibicka, Hansson et al. 2011, Skibicka, Hansson et al. 2012). Kawahara and colleagues showed that hunger in the absence of food creates an aversive neurocircuit in the reward pathway - dopamine outflow in the NAcc shell increased when food was present after injection, however decreased when no food was present (Kawahara, Kawahara et al.

2009). Intraperitoneal administration of ghrelin decreases the firing of dopaminergic neurons in the VTA in food-deprived Wistar rats (van der Plasse, van Zessen et al. 2015). Therefore, peripheral ghrelin induces bimodal effects on the mesolimbic dopamine system depending on the food-consumptive status (Kawahara, Kawahara et al. 2009). For further detailed discussion of the preclinical studies in this area the reader is guided towards recent reviews (Andrews 2011, Perello and Dickson 2015).

There is thus ample evidence to suggest that peripheral ghrelin is able to exert an effect on less accessible brain regions associated with reward and motivation, such as the VTA (Figure 1.5). The mechanism by which peripheral ghrelin achieves access to other subcortical brain areas which are spatially separated from the circumventricular organs has been debated. It is now widely believed that ghrelin itself is not synthesized in the brain (Sakata, Nakano et al. 2009, Furness, Hunne et al. 2011, Cabral, De Francesco et al. 2015). Jerlhag and colleagues have shown that ghrelin is able to access the VTA (Jerlhag 2008), while ghrelin has also been demonstrated to access the hippocampus (Diano, Farr et al. 2006). Since these however, tracer studies using radio-labelled ghrelin have only been able to show that peripheral ghrelin reaches the Arc at the level of the ME (Schaeffer, Langlet et al. 2013), and to a lesser extent the area postrema (Furness, Hunne et al. 2011). An evolutionally developed pathway has been argued to allow for selective transport of ghrelin across the BBB (Banks 2002, Banks, Burney et al. 2008). *In vitro,* human ghrelin exhibits saturable transport mechanics in the blood-to-brain as well as brain-to-blood directions in a rat cerebral microvessel endothelial model (Pan, Tu et al. 2006). An *in vivo* mouse model reported findings consistent with this (Banks 2002). Indeed, many other endogenous substrates have inherited carrier mediated transport systems, such as glucose and insulin (Schwartz, Sipols et al. 1990, Drewes 1998). Furthermore, there is evidence to show that access of ghrelin to the brain via diffusion can increase or decrease depending on the physiological/metabolic backdrop or state of hunger (Banks, Burney et al. 2008). Thus serum factors and physiological state are important determinants in the extent of the saturable ghrelin transport (Banks, Burney et al. 2008). Therefore, it seems that central access of ghrelin may increase in calorie-deprived states.

The most likely mechanism of action of ghrelin in less accessible brain areas however, is through activation of neuronal populations via the permeable zones of the Arc and the area postrema. From here, ghrelin acts to stimulate neuronal projections to other appetite centres not adjacent to the ME, such as the lateral hypothalamus (Olszewski, Grace et al. 2003, Currie, Khelemsky et al. 2012). The LH is a key relay station for neuronal input to the VTA (Nieh, Matthews et al. 2015), and electrical stimulation of the LH induces voracious feeding even in well-fed animals (Stuber and Wise 2016). It receives multiple excitatory and inhibitory inputs from both cortical and subcortical structures, however of particular note is input from the adjacent Arc (Lutter and Nestler 2009). Differentially stimulating the neurons projecting from the Arc to the LH proves that homeostatic energy demands are met by Arc, but the LH is responsible for driving reward-motivated feeding (Stuber and Wise 2016). VTA dopaminergic neurons are modulated by the selectively expressed orexin neuropeptides in the LH (Harris, Wimmer et al. 2005). Thus, the LH and orexins play an important role in food and drug reward behaviours (Aston-Jones, Smith et al. 2010, Cason, Smith et al. 2010). Importantly, elevated peripheral ghrelin levels are known to communicate with the VTA to increase the rewarding value of food in an orexin-dependent manner (Perello, Sakata et al. 2010, Sheng, Santiago et al. 2014). Therefore, in periods of hunger ghrelin is able to access the Arc to stimulate homeostatic feeding, while the LH is concomitantly activated, aided by its close proximity and connections with the Arc. The associated hedonic output is distinct from, yet intertwined with, homeostatic feeding due to its arcuate nucleus-dependant stimulation.

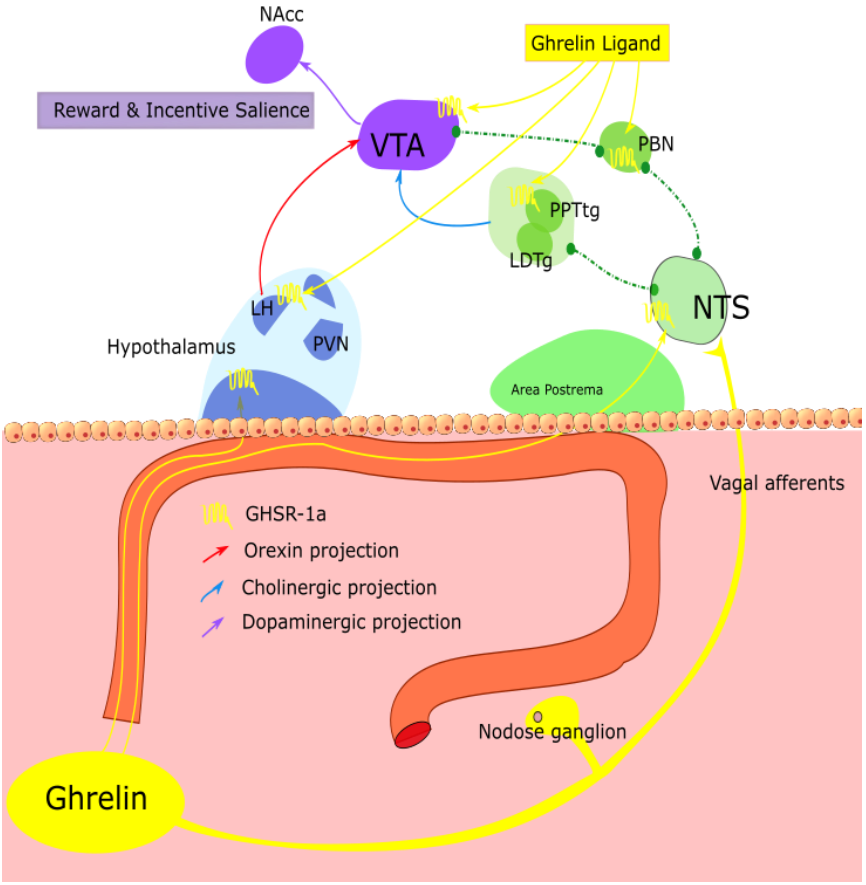



***Figure 1.5. Direct and indirect access of ghrelin to the mesolimbic circuitry*** *The routes by which ghrelin and ghrelin ligands can traverse the blood-brain barrier (BBB). Direct activation of the mesolimbic circuitry can be attained by a centrally penetrant ghrelin agonist or by ghrelin which freely diffuses across the BBB. Indirect activation of mesolimbic circuitry is attained via the homeostatic mechanism through the "leaky" BBB capillaries at the median eminence and the area postrema. Ghrelin signalling initiating in the arcuate nucleus increases the rewarding value of food via orexin projections (red arrow) to the ventral tegmental area (VTA) from the lateral hypothalamus (LH). The nucleus tractus solitarius (NTS) displays connections with the hypothalamus, as well as the parabrachial nucleus (PBN), the laterodorsal tegmental area (LDTg) and pedunculopontine tegmental area (PPTtg), all of which have confirmed roles in either reward signalling (LDTg and PPTtg, blue arrow) or gustatory processes (PBN). Central penetration of ghrelin compounds may act directly on GHSR-1a expressed in these regions to modulate incentive salience of food (purple arrow).*

Another brain area of note for appetite regulation is the parabrachial nucleus, which is located in the hindbrain near the NTS (Saper and Loewy 1980, Cornwall, Cooper et al. 1990, Krukoff, Harris et al. 1993, Grill, Friedman et al. 1995). Like the

Arc, the NTS is spatially located near a permeable or "leaky" area of the BBB and sends glutamatergic signals to the parabrachial nucleus (PBN). Recent work has confirmed this region also receives GABAergic input from hypothalamic agouti-related peptide neurons (Wu, Boyle et al. 2009). The PBN is an important site for processing of gustatory sensory information, with lesions of this area leading to disruption of hedonic feeding and taste-reactivity patterns (Grill, Friedman et al. 1995, Scalera, Spector et al. 1995, Spector, Scalera et al. 1995, Berridge and Robinson 2003). The PBN projects to several areas, notably the LH, paraventricular hypothalamus, and VTA (Jhamandas, Harris et al. 1992, Coizet, Dommett et al. 2010, Oliveira-Maia, Roberts et al. 2011, Abizaid and Horvath 2012). Afferent signals to the paraventricular nucleus of the hypothalamus exist which may be involved in tuning the behavioural response to rewarding food (Igelstrom, Herbison et al. 2010). Interestingly, the parabrachial nucleus itself expresses GHSR-1a and unsurprisingly this hedonic "hotspot" is therefore responsive to ghrelin treatment (Sárvári, Kocsis et al. 2014). Consequently, it is postulated that in periods of hunger plasma ghrelin conveys NTS-dependent signalling to the PBN to exert an effect on feeding and reward behaviour (Skibicka and Dickson 2011, Wu, Clark et al. 2012).

Other areas such as the laterodorsal tegmental area and pedunculopontine tegmental neurons express GHSR-1a and elicit excitatory input to the VTA (Jerlhag 2008, Kim, Nakajima et al. 2009). The pedunculopontine nucleus is implicated in the motivational effects of drugs and food (Lanca, Adamson et al. 2000). Interestingly, *in vitro* work has demonstrated an excitatory effect of ghrelin on pedunculopontine neurons, suggesting a role in food reward (Kim, Nakajima et al. 2009, Kim, Nakajima et al. 2009). The laterodorsal tegmental area increases DA output in the NAcc via the VTA, thereby confirming a GHSR-1a dependant role in reward (Jerlhag, Egecioglu et al. 2006, Jerlhag, Egecioglu et al. 2007).

### 1.8.1 Homeostatic "Gating" of the Reward System

Two decades of research on the effects of exogenous ghrelin has clearly demonstrated the function of GHSR-1a mediated signalling at the level of both homeostatic and non-homeostatic food intake. For homeostatic food intake it is clear

that ghrelin has ready access to sites involved in feeding initiation through permeable brain capillaries and tanycytes (Berthoud 2006), as well as vagal nerve communication (Date, Murakami et al. 2002, Cowley, Smith et al. 2003, Date, Shimbara et al. 2006, Date 2012). Hedonic and motivational aspects of food intake have also been investigated mechanistically through site-specific administration (Jerlhag, Egecioglu et al. 2007, Egecioglu, Jerlhag et al. 2010, Kawahara, Kaneko et al. 2013). The ability of ghrelin to communicate to less accessible GHSR-1a expressing brain areas such as the VTA, LH and parabrachial nucleus suggests an indirect neural mechanism (Cabral, Valdivia et al. 2014). This is indicative of modulation or "gating" of the motivated response for food by systemic signals of energy homeostasis (Ferrario, Labouèbe et al. 2016).

The midbrain reward system is thus heavily dependent on homeostatic appetite regulation in the Arc and NTS, which constitute key "gatekeeping" structures to check the reward system under normal circumstances (Bouret, Gorski et al. 2008). Perello and colleagues confirmed that neural connections between the Arc and the VTA were responsible for peripheral ghrelin's rewarding effect (Perello, Sakata et al. 2010). As we have seen however, preclinical and clinical studies have tended to use supra-physiological doses of ghrelin which may artificially increase delivery across the BBB by saturable transport processes (Banks 2002) and diffusion from the circumventricular organs (Cabral, De Francesco et al. 2015). Elevated endogenous levels of ghrelin are able to elicit the same effects on hedonic aspects of food intake as high exogenous doses. This is due to the synergism of many systemic signals in energy-deprived states. The administration of high doses of a pleiotropic hormone may thus be leading to confounding compensatory mechanisms, particularly in relation to glucose homeostasis (Figlewicz, Evans et al. 2003, Chabot, Caron et al. 2014, Sheng, Santiago et al. 2014). Directly targeting the GHSR-1a expressed in the reward circuitry through enhanced BBB penetration may hold therapeutic potential. One could hypothesise that a centrally-penetrant ghrelin agonist may affect mesolimbic DA levels and incentive valuation of food more directly than non-penetrating ghrelin agonists, or even ghrelin itself, through direct action on the GHSR-1a expressed on the LH, parabrachial nuclei and the VTA.

## Reward system activation: the key role of dopamine

Dopaminergic neurons account for less than a hundredth of the total neuronal population of the brain, however they exert a profound effect on brain function (Arias-Carrión, Stamelou et al. 2010). DA's involvement in motor control, particularly in relation to Parkinson's disease was the subject of original focus. Since then, the molecule's involvement in the neurobiology of psychiatric disorders such as schizophrenia and attention deficit hyperactivity disorder (ADHD) has become increasingly apparent. Not least of these, is the pivotal role which DA plays in the brain's reward system (R A Wise and Rompre 1989). Spatially, dopaminergic neurons are localized to the midbrain, forebrain and olfactory bulbs, although the majority of cells reside in the midbrain. Of particular interest to motivational and hedonic aspects of reward are those located in the VTA, while the nigrostriatal pathway plays the crucial role in voluntary movement control. The mesocorticolimbic pathway can be deconstructed to the mesolimbic and mesocortical pathways, both of which originate in the VTA and modulate emotional behaviours.

DA facilitates the specific consolidation of experiences that are associated with reward, hence assuring repetition through assigning motivational salience to the experience (Arias-Carrión, Stamelou et al. 2010). Basic mechanisms of survival such as eating, drinking and reproduction are fundamentally underpinned by reward system functioning. Essentially, DA facilitates the selective reinforcement of initially random behaviours, which become associated with the attainment of an environmental stimulus. By biasing the consolidation of associations between rewards and otherwise insignificant stimuli, the process of learning occurs. Once the stimulus has been paired with the reward, the association can remain even after the reward has been devalued by the absence of appropriate drive states such as hunger or thirst (Changizi, McGehee et al. 2002), or due to DA system blockade (Dickinson, Smith et al. 2000).

It is in this context that the phenomenon of eating for pleasure, rather than hunger, can be better understood. The rewarding properties of eating, particularly the consumption of palatable foods beyond metabolic demand are largely controlled by dopaminergic signalling within the mesolimbic reward system, which elicits a

primitive drive to overconsume calories to compensate for times of deprivation (Wise 2006, Wang, Volkow et al. 2009, Volkow, Wang et al. 2011, Waterson and Horvath 2015). The initial associations forged by a reward system recognizing the apparent salience of energy-dense foods are robust and long lasting. However, these associations become harmful in the context of readily available high-calorie food in the Western world (Neel 1962). Bart Hoebel and colleagues drew comparisons between the recruitment of DA in the reward system by sugar, and drugs of abuse (Avena, Rada et al. 2008). The concept of food addiction and the role of DA has hence come to the fore in recent years.

## 1.9 Microdialysis as a tool to investigate reward system activation

DA signalling *in vivo* can be monitored in a number of ways – electrophysiologically, via recording of firing activity of the neurons, or by monitoring extracellular concentrations of DA. The latter can be performed using the techniques of microdialysis, voltammetry or brain imaging (e.g. PET) (Di Chiara 1990, Robinson, Venton et al. 2003). The temporal resolution of each of these techniques differs substantially, from milliseconds for electrophysiological recordings to minutes for microdialysis and PET. Essentially, these techniques have been proposed to quantify different modalities of DA signalling and should be interpreted accordingly in the literature.

For tonic measurements, intracerebral microdialysis facilitates the measurement of free (unbound) substances in the extracellular fluid of many tissues. It has traditionally been used in the neuroscience field to quantify endogenous levels of neurotransmitters, such as DA, in the brain extracellular fluid, however the measurement of exogenous substances is also possible with this technique. Of particular use is that microdialysis samples can be collected in real time in conscious, freely-moving animals (Chefer, Thompson et al. 2009). Modern microdialysis techniques were introduced in the 1970's by Ungerstedt and colleagues (Ungerstedt and Pycock 1974, Darvesh, Carroll et al. 2011). The pivotal component of this setup is the microdialysis probe, a small semi-permeable membrane which allows the free diffusion of solutes across from the brain region of interest, to the dialysate within the

probe. The probe is constantly being perfused with dialysate solution which is physiologically identical to the extracellular fluid, normally CSF. The dialysate is constantly perfused at a defined flow-rate using a syringe micropump and hence the recovered analyte elutes out and can be expressed as concentration as a function of time as a result of the intervention.

The active membrane of the probe can be accurately localized to a specific brain region of interest using stereotaxic surgery. At the level of the membrane, molecular diffusion takes place according to Fick's law; the rate of diffusion is directly proportional to the concentration gradient (Bungay, Morrison et al. 1990). The amount of analyte in the perfusate will hence be proportional to the concentration of analyte in the area of interest. The perfusion fluid traverses the probe at a defined flow rate typically 0.5 - 5 $\mu l.min^{-1}$. The outlet tubing allows for collection of the dialysate in an appropriate vial, which is subsequently stored and analysed using a suitable technique such as HPLC. The concentration of the analyte in the dialysate is directly proportional to the concentration in the extracellular fluid surrounding the active site of the probe. However, the dialysate concentration will always be lower than the actual concentration as there is not complete recovery from the probe – typically one would expect ~10% recovery per mm of active membrane window. The relationship between dialysate and periprobe concentrations is termed 'probe recovery' (Anderzhanova and Wotjak 2013). The recovery from each probe largely depends on factors such as perfusion flow rate, size of membrane window, properties of the specific analyte. Probe recovery can be estimated *in vitro* by immersing the probe in a solution of known concentration of analyte and comparing this to the concentration achieved in the dialysate.

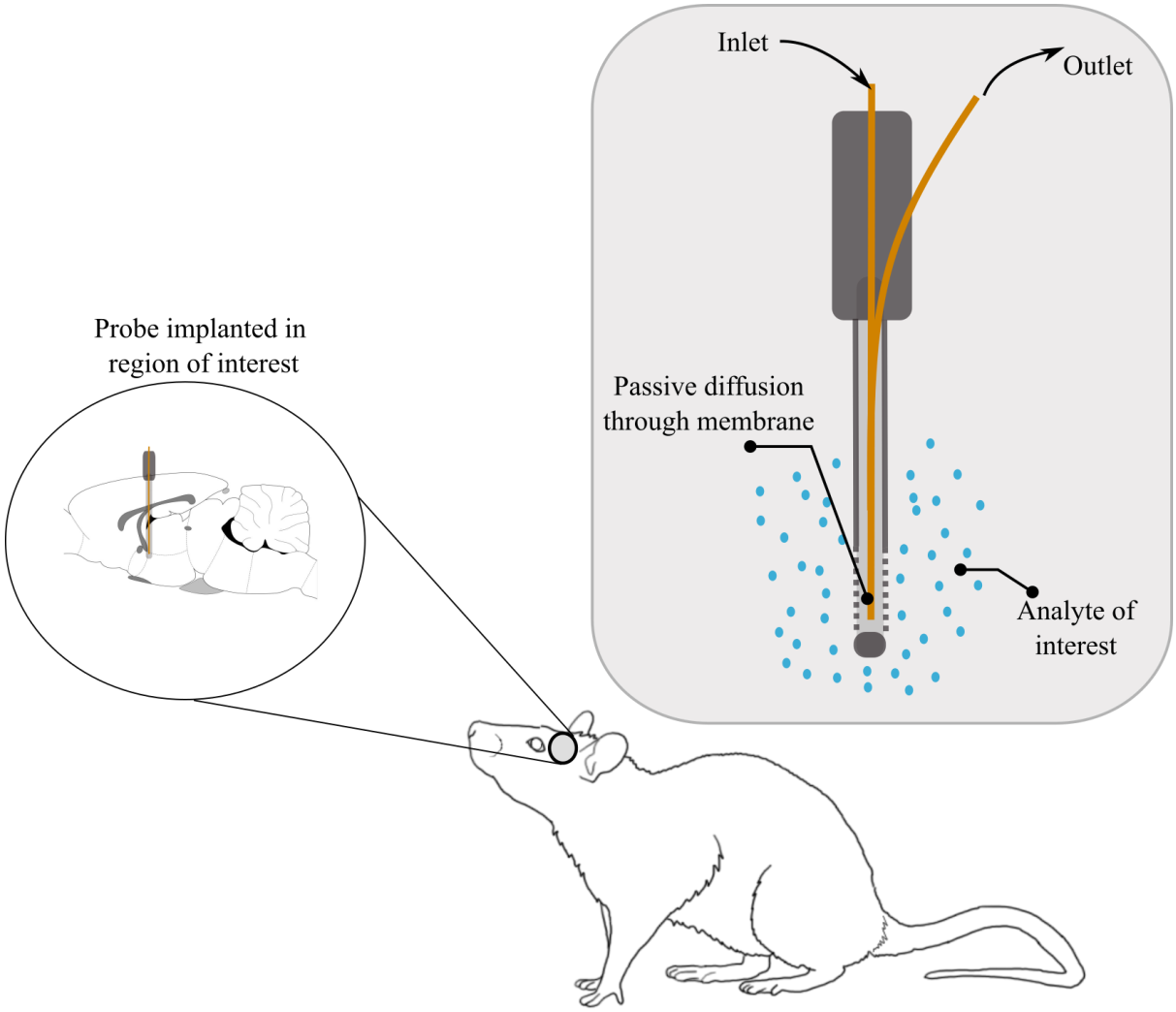



***Figure 1.6. Microdialysis in the conscious, freely-moving rat.*** *The microdialysis probe consists of a semi-permeable membrane at the tip of the probe which allows for free diffusion of analyte of interest into the probe perfusate in a concentration-dependent manner. The probe is inserted through a surgically implanted guide cannula which is implanted in the skull in a stereotaxic surgery.*

## 1.10 Microdialysis, dopamine and ghrelin: current status

Ghrelin is one of the key neurotransmitters which contributes to incentive salience of food via reward system activation (Perello, Sakata et al. 2010, Skibicka and Dickson 2011, Skibicka, Hansson et al. 2011, Schellekens, Finger et al. 2012, Skibicka, Hansson et al. 2012, Skibicka, Shirazi et al. 2013, Perello and Dickson 2015). As discussed above, increases in circulating ghrelin such as those seen in a fasting period, are responsible for signalling an increase in appetite and hunger (Cummings, Purnell et al. 2001). A concomitant increase in the perceived palatability and motivation to work for food is observed. Furthermore, elevated ghrelin levels in the blood are linked with increased dopaminergic activity in the brain; fMRI in human subjects confirms enhanced activation of central reward circuitry induced by pleasurable food images, when these images are preceded by ghrelin administration (Malik, McGlone et al. 2008, Goldstone, Prechtl et al. 2014). Abizaid and colleagues reported that the GHSR-1a is expressed in key nodes of the reward circuitry such as the VTA, and demonstrated that ghrelin binds to VTA neurons, triggering dopaminergic neuronal activity, synaptic plasticity and increase turnover of DA. Ghrelin's role in augmenting the incentive salience of food via the mesolimbic reward neurocircuitry has thus been highlighted (Andrews 2011, Perello and Dickson 2015)

Given the role of the mesocorticolimbic DA system in mediating both the rewarding properties of food intake, and the motor stimulation caused by food-seeking behaviour, a series of microdialysis publications investigated the effects of ghrelin on DA output in the midbrain reward circuitry. Previously published data showed that central administration of ghrelin modulates the *in vivo* DA levels in the NAcc (Jerlhag 2006 & 2007). Furthermore, it was reported that intra-VTA administration of ghrelin initiates feeding which can be subsequently blocked with a ghrelin antagonist. Jerlhag and colleagues however, were the first to investigate ghrelinergic-manipulation of the mesolimbic pathway using *in vivo* microdialysis. Moreover, a GHSR-1a knockout model to show that DA output in the NAcc elicited by rewarding food is GHSR-1a dependent (Egecioglu, Jerlhag et al. 2010). Kawahara and colleagues also used microdialysis to describe the food-dependent effects of accumbal DA outflow (Kawahara, Kawahara et al. 2009).

# Disorders of appetite: Current Status and Implications for ghrelin therapy

Consequences of over- and under-eating constitute ever-expanding health problems that remain unanswered in modern society, despite education, public health campaigns and pharmacotherapy (Schellekens, Dinan et al. 2010, von Haehling and Anker 2014). Thus, there is an impetus to understand the physiological mechanisms underlying central appetite regulation and food intake in order to design novel treatment strategies for eating disorders. However, despite almost 20 years since it's discovery by Kojima and colleagues, no specific ghrelin targeting anti-obesity drug or cachexia therapeutics are on the market for clinical use (Kojima, Hosoda et al. 1999). The literature on ghrelin illustrates a plethora of information, yet we are still faced with a paucity of success. As knowledge on ghrelin increased, the role of the hormone shifted from the key protagonist in feeding initiation to be considered as part of a spectrum of diverse physiological processes. The peripheral and central distribution of the GHSR-1a and the heterogenous nature of GHSR-1a signalling result in pleiotropic actions of ghrelin, many of which are still being investigated.

Food intake and incentive valuation of food are centrally-mediated processes. Ghrelin or ghrelin ligands can access the brain from the periphery by circumventing the BBB at permeable locations adjacent to homeostatic appetite centres, and indirectly influence reward centres through neural connections stemming from these areas (Perello, Sakata et al. 2010, Ferrario, Labouèbe et al. 2016). The importance of GHSR-1a signalling in the mesolimbic dopaminergic pathway as a barometer for the incentive salience of food has been well described. However, the action of GHSR-1a signalling on reward areas is closely intertwined with homeostasis and is regulated in this respect (Cabral, De Francesco et al. 2015, Ferrario, Labouèbe et al. 2016). The peripheral metabolic confounders in systemic ghrelin therapy, particularly relating to glucose homeostasis, may be contributing to the lack of successful preclinical moieties translating to clinical practice (Su, Geng et al. 2016). BBB-penetrant ghrelin agonists should bypass the homeostatic "gating" at the level of the Arc and NTS. This means that they would act directly on GHSR-1a in less accessible brain areas associated with

motivation and incentive valuation of food, such as the LH and VTA. Since the decision to eat is consciously made based on perceived palatability, centrally penetrating ghrelin agonists or indeed antagonists, could prove successful in manipulating top-down regulation of food intake.

## 1.11 Pre-cachexia and Cachexia

The hypothalamic neural circuits controlling energy balance are well described above in Section 1.1. Dysregulation of these mechanisms in ageing can lead to conditions of undereating and malnutrition, and appetite decline in the elderly is an important consideration for the healthcare industry (Chapman 2004, Hickson 2006, Malafarina, Uriz-Otano et al. 2013). Average life-expectancy has increased dramatically with recent years, due to better healthcare and nutrition. Conversely, appetite and food intake decrease with the normal ageing process. A number of physiological changes occur during ageing which disrupt the mechanisms of energy homeostasis and lead to reduced appetite and food intake, resulting in malnutrition and the loss of lean body mass (Evans, Morley et al. 2008, Stoyanova 2014). A resultant increased risk of acute or chronic illness, hospitalization and loss of independence leads to a greater burden on community and medical services. A ubiquitous decline in ghrelin levels is thought to be a major contributory factor to appetite reductions and weight loss (Chapman 2004, DeBoer 2008). This age-related phenomenon is dubbed the "somatopause" and results in decreased lean body mass (sarcopenia), frailty, and are linked to cardiovascular issues, as well as cognitive and sleep disorders. Moreover, the incidence of chronic conditions increases dramatically in older demographics, with an increased incidence of chronic diseases such as cancer, chronic obstructive pulmonary disease (COPD), congestive heart failure (CHF) and chronic kidney disease (CKD) – it is estimated that 80% of older adults suffer from at least one chronic condition. These conditions are known to disrupt the homeostatic energy regulation by decreasing appetite and increasing energy expenditure in patients.

These physiological changes can result in the onset of pre-cachexia, defined as the presence of all the following criteria: (a) underlying chronic disease; (b) unintentional weight loss 5% of usual body weight during the last 6 months; (c) chronic or recurrent systemic inflammatory response; (d) anorexia or anorexia-related symptoms. In elderly patients, poor nutritional status in elderly patients hence complicates, and is complicated by, chronic diseases and is known to result in prolonged hospital stays, lessened independent living and poorer response to treatment, leading to an overall greater burden on global health infrastructures and

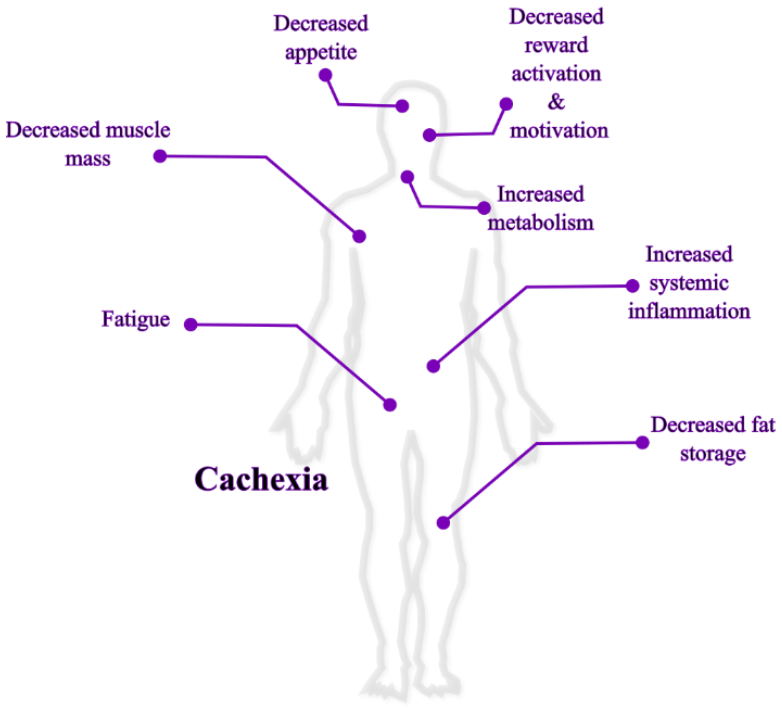
lower clinical outcomes (Malafarina, Uriz-Otano et al. 2013). Age-related malnutrition coupled with chronic illnesses cause a cascade of metabolic changes resulting in loss of lean and fat mass, and the development of cachexia (Chapman 2004, DeBoer 2011, Malafarina, Uriz-Otano et al. 2013). Therefore, progression of illness and malnutritive status can result in cachexia, a multifactorial syndrome defined by an ongoing loss of skeletal muscle mass and functional impairment, that cannot be fully reversed by conventional nutritional support.

Pharmacological strategies for increasing appetite in cachexia spans the use of corticosteroids, cannabinoids, megestrol and progestogens, thalidomide and gastrointestinal prokinetics (Radbruch L 2010, Aoyagi, Terracina et al. 2015). To date although promising results have been demonstrated these therapies are not without drawbacks. Caveats to current treatment strategies such as the above have been reviewed extensively (Argilés, López-Soriano et al. 2008, Evans, Morley et al. 2008, Argilés, López-Soriano et al. 2013, De Ng, Bruera et al. 2016).

### 1.11.1 Neuronal alterations in cachexia

Onset of cachexia is associated with a general increase in systemic inflammation and cytokine release, culminating in an increase in the basal metabolic rate and energy expenditure (Figure 1.7) (DeBoer 2011). Cancer and other chronic conditions also result in an increase in cytokine release, which are known to act on the central nervous system to alter the release and function of a plethora of neurotransmitters In particular, the hypothalamus is a major target for inflammatory cytokines (Grossberg, Scarlett et al. 2010). Neuronal inflammation, as reported by NF-κB activation, is localized almost exclusively to the hypothalamus and brainstem following a systemic immune insult (Laflamme and Rivest 1999). This may be due to the permeable BBB at the level of the median eminence which affords access of blood-borne signals to the central compartment. Neuronal response to inflammation results in local cytokine production in appetite centres, many of which express receptors for the same cytokines. Immediate early gene-activation as shown by c-Fos nuclear staining is detected in the Arc and paraventricular nucleus in response to peripheral inflammatory stimuli (Wan, Janz et al. 1993, Elmquist, Scammell et al.

1996). Therefore, anorexigenic effects of systemic inflammation are exerted through the hypothalamic centres responsible for energy balance. Feeding nuclei in the brainstem such as the NTS also display a robust induction of c-Fos and NF-κB in response to peripheral inflammation (Grossberg, Scarlett et al. 2010).



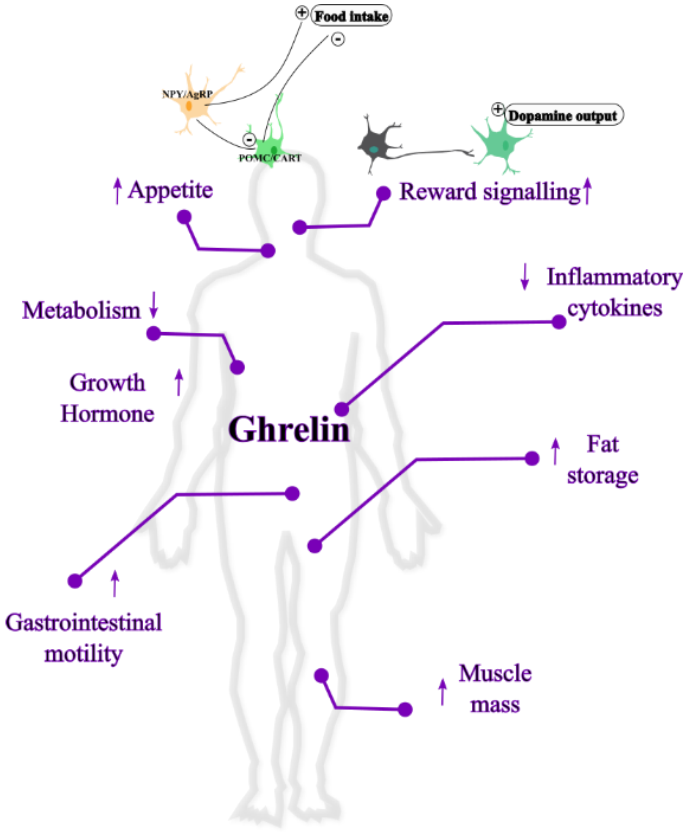



***Figure 1.7. Physiological alterations in cachexia and potential of ghrelin therapy:*** *The onset of cachexia is associated with various deleterious effects on appetite, metabolism, reward processing and systemic inflammation which results in a net catabolic effect in the body. Ghrelin exerts antagonistic effects to these. The hormone is orexigenic and somatotrophic, while also stimulating increases in gastrointestinal motility and reward system activation and decreasing systemic inflammation.*

Ghrelin treatment results in an increase in the expression and release of orexigenic agouti-related peptide (AgRP) and neuropeptide-Y (NPY), with a concomitant decrease in the expression of pro-opiomelanocortin (POMC). Downstream effects of this activation lead to increased food-seeking behaviour and a decrease in resting energy expenditure. Recent reviews have discussed the merits and limitations to date of ghrelin therapy (DeBoer 2008, DeBoer 2011). There are also various extra-hypothalamic effects of ghrelin pertaining to cachexia; specifically, reduced inflammation, cardiovascular effects, increase fat storage, gut motility and blood glucose homeostasis during fasting. Critically, the reward system is capable of overriding the hypothalamic "homeostatic" system of food intake regulation. This results in the intake of calorie-dense, palatable food which is beyond that needed to

satisfy metabolic demand. It is known that motivation to seek out and consume food, as well as the perceived palatability of food is very low in cachectic states (Evans, Morley et al. 2008). Despite this, relatively little has been carried out on the impact of cachectic states on these important pathways. Immunohistochemistry of the NAcc, caudate putamen and other ventral striatal structures revealed FosB-positive neurons and/or prodynorphin or proenkephalin mRNA during cachexia-like states (Pourtau, Leemburg et al. 2011). The decision to eat is largely a top-down decision made through input of visual, gustatory, olfactory and emotional stimuli – these findings indicate that forebrain structures that are part of these decision-making networks are altered in tumour-associated cachexia syndrome and may contribute to the lack of compensatory eating in response to weight loss, a hallmark of this condition.

## 1.12 Obesity, anorexia and binge eating disorders

The primitive drive to overconsume calories in times of abundance in order to deal with long periods without food was a useful survival tool. However, this has become a redundant trait in the last century with the abundance of readily-available food in Western society (Neel 1962). Calorie overconsumption coupled with increasingly sedentary lifestyles has led to an obesity epidemic (WHO 2018). Synonymous with increased morbidity and mortality, obesity is widely seen as the largest and fastest growing public health concern of modern times (Isomaa, Almgren et al. 2001, Ng, Fleming et al. 2014, Martin, Mani et al. 2015, Seidell and Halberstadt 2015, Tremmel, Gerdtham et al. 2017). As discussed in detail above, there are highly conserved neural pathways which exist to promote the consumption of calories surplus to metabolic requirements (Section 1). Those foods which are high in calories, typically palatable sugary foods, are potent instigators of reward system activation that trigger robust and long-lasting learned associations between the stimulus and reward. This leads to an overly-primed reward system which in turn exhibits increased anticipatory processing of rewards yet less pleasure is attained from the attainment of the reward (Volkow, Wang et al. 2012). In this context, the fundamental susceptibility of humans to the overconsumption of high-fat and high-sugar meals can be better understood. Conversely to cachexia, the underlying issues with obesity is not down to a malfunctioning reward system but rather societal and cultural predispositions to food

rewards which prime an effective neuronal pathway to promote overconsumption (Neel 1962, Waterson and Horvath 2015). Alterations in striatal dopaminergic signalling are thought to be a causative mechanism facilitating hyperphagia (Wise 2006). Obese subjects show increased neural activity in reward and motivation circuitry when presented with pleasurable food images (Stoeckel, Weller et al. 2008). Indeed, it was found that activation of the NAcc was negatively correlated with body weight in these studies (Killgore and Yurgelun-Todd 2005). This suggests that the more food is consumed, the more sensitive the reward system is to pleasurable food images. The reactivity of obese neurocircuitry to food is thereby enhanced. Furthermore, it was found that obese individuals needed to consume more of the same food to get an equal consummatory reward, yet there is a comparative increase in the activation of cortical regions which process the anticipation of reward (Stice, Spoor et al. 2008, Stice, Yokum et al. 2010). In other words, there is enhanced anticipation and motivation to obtain the reward, but less pleasurable consequences to obtaining it. Thus over-anticipation and under-appreciation promotes overconsumption of food to redress the imbalance (Volkow, Wang et al. 2012).

Anorexia nervosa (AN) and binge eating (BE) disorders warrant mention here also. A large body of literature has linked dysregulation in reward systems with eating disorders such as AN and BE, where there is a fundamental issue with food reward valuation and learning via their interactions with the mesolimbic dopamine system. Although the physiological mechanisms underlying these conditions are incompletely understood, a strong genetic predisposition has been reported (Cuesto, Everaerts et al. 2017, Berner, Brown et al. 2018). These conditions have traditionally been treated as psychiatric disorders given the high prevalence of comorbid anxiety and obsessive compulsive disorder (OCD) in these patients (Walter H. Kaye, Cynthia M. Bulik et al. 2004, Schalla and Stengel 2018). Though the prevalence of AN and BE are much lower than that of obesity, the impact to quality of life is considerable (Erskine, Whiteford et al. 2016). Indeed, AN is the psychiatric disorder with the highest degree of mortality (Walter H. Kaye, Cynthia M. Bulik et al. 2004, Agh, Kovacs et al. 2016).

Many agents that were heralded as the answer to the obesity problem were subsequently withdrawn owing to an unacceptable burden of side-effects. A number

of centrally acting sympathomimetics such as ephedrine derivatives and phentermine were withdrawn due to concerns over abuse potential and cardiovascular safety (Colman 2005). The serotonergic agent fenfluramine, the monoamine uptake inhibitor sibutramine and the CB1 antagonist rimonabant were withdrawn for links to cardiac issues (fenfluramine and sibutramine) and psychiatric problems (rimonabant)(Weintraub, Sundaresan et al. 1992, Luque and Rey 2002, Kirkham 2009). For AN and BE, cognitive behavioural therapy (CBT) is the mainstay of treatment, with pharmacological management of comorbid symptoms such as anxiety, depression etc. (Walter H. Kaye, Cynthia M. Bulik et al. 2004, Halmi 2005). The altered feeding component is but one visible symptom of a complex neuropsychosocial disorder which is only in the process of being fully understood (Halmi 2013, Cuesto, Everaerts et al. 2017).

## 1.13 Ghrelin as a pharmacological approach for disorders of appetite

The neural network controlling food intake has proven to be one of the most deceptively complex machineries to manipulate. Ghrelin, when discovered in 1999, was heralded as the key to pharmacological manipulation of appetite and body weight. This endogenous hormone has become synonymous with research efforts in the appetite modulation field due to its key position in the mammalian neuraxis controlling energy balance, as a peripherally accessible hormone with centrally-mediated effects on appetite (Horvath, Diano et al. 2001). Ghrelin exerts a number of somatotropic and anti-inflammatory effects (Figure 1.7) and has shown promising results in clinical trials for CACS (Neary, Small et al. 2004, Nagaya, Kojima et al. 2006). Moreover, the synthetic ghrelin agonist anamorelin has shown promising results pre-clinically and clinically and is currently under regulatory consideration for this indication (Pietra, Takeda et al. 2014, Garcia, Boccia et al. 2015). Conversely, ghrelin's role in the reward system has led to it being considered as a therapy for conditions of dysregulation of food reward such as obesity (Horvath, Castaneda et al. 2003). Elevated ghrelin in hunger increases the perceived palatability and motivation to work for a food reward, via mesolimbic system activation (Egecioglu, Jerlhag et al. 2010, Egecioglu, Skibicka et al. 2011). While ghrelin antagonists have shown promising results in pre-clinical

studies (Asakawa, Inui et al. 2003), there is little evidence of sustained anorexigenic properties clinically. Furthermore, the pathophysiology of eating disorders is incompletely understood at present and ghrelin's role in this respect remains unclear (Frank 2013, Cuesto, Everaerts et al. 2017, Schalla and Stengel 2018). A paradoxical elevation of ghrelin in AN has been described despite patients having no drive to consume food (Nedvidkova, Krykorkova et al. 2003, Monteleone, Serritella et al. 2008). Moreover, patients are refractory to ghrelin therapy (Broglio, Gianotti et al. 2004, Miljic, Pekic et al. 2006, Ogiso, Asakawa et al. 2011). Many of these studies however have ignored the acylation status of ghrelin (Ogiso, Asakawa et al. 2011). Des-acyl ghrelin is an important consideration and likely has important clinical sequelae, given that certain studies have found it to have contrasting effects to acyl-ghrelin (Asakawa, Inui et al. 2005) and that there are documented differences in these in AN patients (Koyama, Yasuhara et al. 2010). Furthermore, genetic polymorphisms in the ghrelin and/or GOAT may hold the key to a therapeutic breakthrough given the association with increased prevalence of AN (Dardennes, Zizzari et al. 2007, Muller, Tschop et al. 2011).

Targeting of the ghrelin system has high potential in the treatment of disorders of dysregulation in reward processing. Ghrelin is a contributor to reward system priming and promotes the incentive salience of food (Naleid, Grace et al. 2005, Skibicka and Dickson 2011, Schellekens, Finger et al. 2012, Perello and Dickson 2015). Consequently, antagonizing this system has become a target for the overt activation of the mesolimbic pathway which causes, and is caused by, the overconsumption of palatable foods. Furthermore, ghrelin is part of the complex interplay of genetic and neurobiological factors underlying the pathogenesis and maintenance of eating disorders. Although it's role in this respect is not as well understood, there is a growing body of evidence to suggest that although the etiology of these disorders may not be linked to ghrelin there may be a role for manipulation of the ghrelin axis in such patients (Yi, Heppner et al. 2011, Atalayer, Gibson et al. 2013).

## From pharmaceutical to nutraceutical – opportunities for early intervention in cachexia.

Current pharmaceutical therapy is limited to those patients diagnosed with cachexia and whom are already in a treatment-refractive state. There is growing interest in the area of nutraceuticals as prophylactic or complementary therapies for various illnesses (Santini, Tenore et al. 2017). The potential health benefits of the bioactive fragments which exist in many food and dietary proteins have long been known (Nongonierma and FitzGerald 2015). As such, a pre-emptive nutraceutical approach to treat pre-cachexia has been proposed to augment a weakening ghrelin axis in elderly and infirm cohorts (Howick, Wallace-Fitzsimons et al. 2018).

Nutraceuticals or functional foods are a relatively new concept which sits at the interface of drugs and food. They may be defined as a "food or part of a food that provides benefits to health in addition to its nutritional content". Nutraceuticals and functional foods adopted in the diet may aid in the prevention, or delaying the onset of, pathological conditions (da Costa 2017). Furthermore, they may provide an avenue to potentially delay initiation of pharmaceutical medicines in subjects with milder symptoms. In this respect, the phenomenon of age-related appetite reduction and pre-cachexia is an important, unmet, clinical need (Chapman 2004). The initiation of routine pharmaceutical therapy is imprudent in many cases. However, the impact of poor nutrition on prognosis of co-morbid conditions, as well as on overall quality of life and independent living means that appropriate early interventions are needed (Malafarina, Uriz-Otano et al. 2013). Given the lack of suitable pharmacotherapy and the growing role of nutraceutical science, evidence-based dietary interventions to help delay the onset of a cachectic state due in comorbid illnesses may be a useful avenue. The pro-active targeting of the ghrelin system with dietary-derived bioactives may precede or supplement pharmacological treatment of clinically significant appetite reductions (Howick, Wallace-Fitzsimons et al. 2018).

## 1.14 Dairy-derived dietary bioactives

Recent times have seen an increasing move towards harnessing the health promoting benefits of dairy-derived dietary constituents while providing scientific evidence to substantiate their claim (Hartmann and Meisel 2007). The utilisation of dairy-derived bioactives in appetite-related disorders is now becoming increasingly apparent (Nongonierma and FitzGerald 2015, Torres-Fuentes, Schellekens et al. 2015). In particular, the potential for bioactive protein hydrolysates and peptides to enhance health in conjunction with conventional pharmaceutical therapy is being investigated. Milk has been identified as one of the richest sources of bioactive fragments and there is a growing body of evidence that these can have positive effects on appetite and metabolism (Phelan and Kerins 2011, Schellekens, Nongonierma et al. 2014, Torres-Fuentes, Schellekens et al. 2015, Nilaweera, Cabrera-Rubio et al. 2017). Dairy-derived proteins have been shown to contain bioactive peptide sequences with various purported health benefits, with effects ranging from the digestive system to cardiovascular circulation, immune system and central nervous system. Peptides fractions have been isolated with ACE-inhibitory action, and blood-pressure lowering properties of these dairy-derived bioactives *in vivo* have been reported. Interestingly, the ability of dairy proteins to modulate metabolism and appetite has recently been reported. The ability of a dairy bioactive to enhance satiety and decrease food intake *in vivo* has been shown (Schellekens, Nongonierma et al. 2014). Conversely, recent work has also shown another whey protein isolate to reduce the expression of satiating genes in the hypothalamus and increase food intake in rodents (Nilaweera, Cabrera-Rubio et al. 2017). Furthermore, a casein-derived bioactive fraction with specific serotonin-2C receptor (5-$HT_{2C}$) agonist activity eliciting satiating properties in a rodent model has been described (Schellekens, Nongonierma et al. 2014).

Bioactives that augment the ghrelin system have previously yielded anecdotal evidence of increased appetite, which has since been substantiated by animal and human studies. Rikkunshito (RKT), a long-standing traditional Japanese herb has the ability to function as a ghrelin agonist (Fujitsuka and Uezono 2014). RKT has been shown to reduce weight loss and increase food intake in mouse models of wasting

syndrome (Terawaki, Sawada et al. 2014, Tsubouchi, Yanagi et al. 2014), while a retrospective analysis of cancer patients showed increases median survival time in patients receiving concomitant RKT with their treatment (Fujitsuka and Uezono 2014). Chin-shin oolong tea, a popular tea in Taiwan, was empirically perceived to induce hunger, and subsequently was shown to increase food intake in rats (Lo, Chen et al. 2014). *In vitro*, an isolate from Emoghrelin Heshouwu, a Chinese traditional medicine, was shown to activate the ghrelin receptor and stimulate GH secretion *in vitro*, supporting a claim for its perceived therapeutic efficacy as an anti-aging supplement (Lo, Chen et al. 2015). Furthermore, work in our lab has described ghrelinergic bioactives derived from natural sources (Pastor-Cavada, Pardo et al. 2016, Torres-Fuentes, Pastor-Cavada et al. 2018).

Naturally-derived ghrelin bioactives have clearly demonstrated anecdotal and experimental evidence of efficacy on food intake and gut motility. Dairy-derived peptides are increasingly recognized for their bioactive components which may bestow clinical benefits in the area of appetite and metabolism (Hartmann and Meisel 2007, Torres-Fuentes, Schellekens et al. 2015). However, there exists a major knowledge gap to realizing the full potential of milk protein derived peptides in this respect. Identification and isolation of these bioactives, as well as elucidating their pharmacodynamic parameters are necessary to transfer their potential benefits into functional applications (Korhonen and Pihlanto 2003). An urgent need exists for the development of integrated, multidisciplinary research platforms to address the role and mechanism of action of milk protein-derived peptides in humans (Nongonierma and FitzGerald 2015).

### 1.14.1 Oral peptide delivery – Formulation and release perspectives

One of the research modalities critical to the success of bioactive peptides and other labile substances is pharmaceutical science (Brayden and Baird 2013, Gleeson, Ryan et al. 2016). There is undoubtedly an increasing amount of research on peptides, protein hydrolysates and other dietary-derived bioactive substances with a plethora of biological activities. However, there are many obstacles to the successful

commercialization of these products, not least being the development of appropriate oral delivery systems to protect the bioactivity and physicochemical properties of the payload (McClements, Decker et al. 2009).Without appropriate delivery systems, promising nutraceuticals and bioactives are unlikely to provide the intended physiological benefit due to the various degradative barriers encountered *in vivo*, in addition to barriers to absorption from the gastrointestinal tract (Brayden and Baird 2013, Howick, Alam et al. 2018, Howick, Wallace-Fitzsimons et al. 2018). All of this, in addition to the need for cost-effective strategies for industrial scale-ups, means that the nutraceutical industry would benefit greatly from the experience of traditional pharmaceutical formulation perspectives to yield appropriate encapsulation platforms.

Oral peptide delivery remains a bottle-neck in the transition of potentially effective therapeutics from bench to bedside (Brayden and Alonso 2016). Bioavailability of peptides is consistently poor due to the acidic and enzyme-mediated degradation in gut lumen, leading to loss of efficacy. The rapid degradation of bioactive peptide structures *in vivo* necessitates drug delivery technologies which protect the payload in the gastric compartment and allow for site specific delivery to the small and large intestine (Malik, Baboota et al. 2007). Indeed, oral peptide delivery has been the subject of intense research across the pharmaceutics field, with various approaches adopted to increase bioavailability and limited breakdown. Various formulation approaches have been adopted to protect peptides from degradation within the gastrointestinal tract and increase oral bioavailability, ranging from standard formulations containing functional excipients, to micro- and nano- based (colloidal) delivery systems (Lakkireddy, Urmann et al. 2016). These range from the use of absorption enhancers, enzyme inhibitors and mucoadhesive polymers, to the use of various formulation vehicles and cell penetrating peptides. However, commercial success in terms of an orally active peptide formulation has been limited to a few niche, high potency peptides which can achieve therapeutic efficacy with limited bioavailability (i.e. <1%) (Aguirre, Teijeiro-Osorio et al. 2016) Although limited success has been reported to date there have been a number of interesting developments in recent years (Table 1.3)..

***Table 1.3. Summary of selected clinical approaches to enhance oral peptide delivery*** *Drug delivery system approaches to enhance oral peptide.*

| **Drug delivery system** | **Highlights** | **Protein/peptide** | **Status** | **Reference** |
|---|---|---|---|---|
| Peptelligence™ | • Enteric coated tablet for intestinal release.<br>• Citric acid reduce intestinal pH microenvironment to prevent protease degradation of payload.<br>• Acyl-carnitine used as a tight junction loosener. | Salmon Calcitonin | Phase III complete.<br>Commercially available | (Stern, Mehta et al. 2013) |
| Mycapssa® | • Transient Permeability Enhancer platform<br>• Lipophilic suspension of drug and an absorption enhancer (e.g sodium caprylate or medium chain fatty acid) | Octreotide | Phase III | (Biermasz 2017) |
| Oramed™ | • Protein Oral Delivery (POD™) technology containing a protease inhibitor and an absorption enhancer (EDTA). | Insulin, GLP-1 | Phase II | (Werle, Makhlof et al. 2009) |

| | | | | |
|---|---|---|---|---|
| GIPET® | • Based on the use of medium-chain fatty acids, in particular sodium caprate, which is claimed to open tight junctions transiently. | Insulin, GLP-1 | Phase II | (Halberg, Lyby et al. 2019) |
| Eligen® | • SNAC (sodium salcaprozate) and 5-CNAC (N-(5-chlorosalicyloyl)-8-aminocaprylic acid) used as absorption enhancers to enhance solubility of poorly permeable macromolecules. | Vitamin $B_{12}$<br><br>Semaglutide | Commercially available<br><br>Phase II | (Maher, Brayden et al. 2019) |
| NOD® | • Bioadhesive calcium phosphate nanoparticles | Insulin | Phase I | (Mathur, Mathur et al. 2018) |
| PharmaFilm® | • Surface modified gold nanoparticles complexed with peptide and embedded into a mucoadhesive film for buccal delivery. | Insulin | Phase II | (Hassani, Lewis et al. 2015) |
| Oral-lyn™ | • Micellar solution buccal spray combined with permeation enhancers, bile salts and sodium caprate. | Insulin | Phase II | (Hassani, Lewis et al. 2015) |

# Thesis objectives

Regulation of appetite and energy balance is achieved via a complex array of neurobiological signals spanning the gut-brain axis, communicating reciprocally to maintain homeostasis. Disorders of appetite are responsible for significant morbidity and mortality. Ghrelin is the only known peripheral orexigenic hormone and as such holds therapeutic promise in the treatment of both under- and over-eating, however currently no ghrelinergic agents have reached the market. This thesis focuses on two distinct areas of research; firstly, the targeting of GHSR-1a using dietary peptides for early stage treatment of appetite disorders, and secondly, the investigation of biased signalling and biodistribution of synthetic ghrelin ligands to enhance the functional efficacy of GHSR-1a ligands.

***Aim 1:*** *Investigate dairy-derived hydrolysate ability to activate GHSR-1a.*

Milk is one of the largest repositories for bioactive peptides, with numerous purported health benefits (Phelan and Kerins 2011). The potential applicability of dairy-derived bioactives in appetite-related disorders is becoming increasingly apparent (Nongonierma and FitzGerald 2015, Torres-Fuentes, Schellekens et al. 2015, Nilaweera, Cabrera-Rubio et al. 2017). GHSR-1a is a key peripherally-accessible target for appetite modulation. *In vitro* investigations into the ability of novel dairy hydrolysates to activate the GHSR-1a will be undertaken in order to assess their potential for a dietary based therapeutic in disorders of appetite (Chapter 2 & 3).

***Aim 2:*** *Develop an appropriate gastro-protected formulation for oral delivery.*

Given the labile nature of peptide-based bioactives, appropriate encapsulation strategies are needed in order to translate *in vitro* potency from bench to bedside (Brayden and Baird 2013, Brayden and Alonso 2016, Gleeson, Ryan et al. 2016). Therefore, in order to realize a clinically viable bioactive product, appropriate steps must be taken in order to deliver the ghrelinergic bioactives from Aim 1 intact to the intended site of action. A gastro-protected pellet formulation will be developed which

will protect the peptide payload from acid-denaturation *in vivo* while also being amenable to dosing in small animal studies (Chapter 2 & 3).

***Aim 3:*** *Assess food intake in rodents after dosing with novel hydrolysate.*

Much nutraceutical and bioactive research to date lacks tangible *in vivo* evidence of effect. Proof-of-concept studies are needed in order to ascertain if *in vitro* bioactivity can translate to an effect in a physiological model. Therefore, food intake in a rodent model will be assessed after dosing of ghrelinergic hydrolysates under different modes of administration (Chapter 2 & 3), with the ultimate aim of enabling clinical studies to be carried out as part of the Food for Health Ireland research collaboration.

***Aim 4:*** *Investigate biased signalling of synthetic ghrelin ligands on GHSR-1a.*

Growing evidence points to the significance of biased signalling of ghrelin ligands in exerting differential effects *in vivo* (M'Kadmi, Leyris et al. 2015, Mende, Hundahl et al. 2018, Ramirez, van Oeffelen et al. 2018). Largely ignored until recently, it is now thought that the ability of GHSR-1a ligands to preferentially activate varying downstream pathways may lead to the development of more effective, selective ghrelinergic therapies. Therefore, various cell-based assays will be utilized to compare and contrast the downstream signalling of two novel ghrelin ligands, anamorelin and HM01 in order to further characterize their pharmacodynamics (Chapter 4).

***Aim 5:*** *Investigate the effects of novel ghrelin ligands on appetite and reward motivated behaviours.*

The mesolimbic circuitry is a key driver for reward-based feeding and may represent an underexploited machinery to manipulate food intake (Howick, Griffin et al. 2017). The GHSR-1a is expressed in key nodes of the reward system such as the VTA. Consequently, biodistribution of ghrelin ligands is increasingly recognized as an important determinant for *in vivo* efficacy based on ability to gain access to the CNS and reward areas (Howick, Griffin et al. 2017). Various appetite and reward motivated behavioural paradigms will be compared for two novel, synthetic GHSR-

1a agonists, anamorelin (non-BBB penetrant) and HM01 (BBB penetrant) in order to bolster the theory that central penetrance is an important determinant of *in vivo* potency.

**Aim 6:** *Investigate underlying mechanisms using c-Fos immunostaining and in vivo microdialysis.*

The neural mechanisms underlying appetite and reward behaviours after treatment with ghrelin have been well established. However, ghrelin's ability to effect changes to the mesolimbic circuitry despite being limited to the periphery *in vivo* remains a point of debate in the literature (Cabral, De Francesco et al. 2015, Edwards and Abizaid 2017). Mechanistic investigation using c-Fos as a marker of neuronal activation *ex vivo* will be undertaken after treatment with anamorelin (non-BBB penetrant) and HM01 (BBB penetrant). Furthermore, extracellular DA levels in the NAcc will be quantified using *in vivo* microdialysis studies in conscious, freely feeding rats in order to delineate differences between anamorelin and HM01.

# Chapter 2

# A novel dairy-derived hydrolysate modulates food intake via ghrelinergic signalling *in vivo*.

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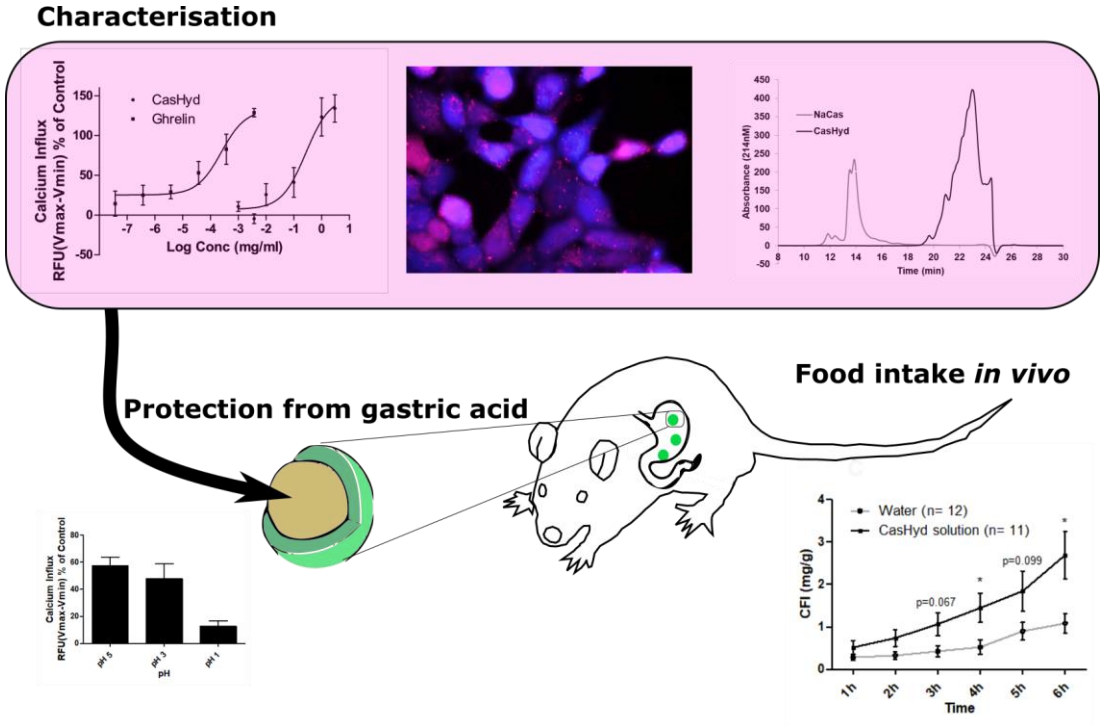
# Abstract

Recent times have seen an increasing move towards harnessing the health promoting benefits of food and dietary constituents while providing scientific evidence to substantiate their claim. In particular, the potential for bioactive protein hydrolysates and peptides to enhance health in conjunction with conventional pharmaceutical therapy is being investigated. Dairy-derived proteins have been shown to contain bioactive peptide sequences with various purported health benefits, with effects ranging from the digestive system to cardiovascular circulation, immune system and central nervous system. Interestingly, the ability of dairy proteins to modulate metabolism and appetite has recently been reported.

The ghrelin receptor (GHSR-1a) is a G-protein coupled receptor which plays a key role in the regulation of food intake. Pharmacological manipulation of the GHSR-1a receptor has therefore received a lot of attention as a strategy to combat disorders of appetite and body weight, including age-related malnutrition and the progressive muscle wasting syndrome known as cachexia. In this study, a novel milk protein-derivative is shown to increase GHSR-1a-mediated intracellular calcium signalling in a concentration-dependent manner *in vitro*. Significant increases in calcium mobilization were also observed in a cultured neuronal cell line heterologously expressing the GHS-R1a. In addition, both additive and synergistic effects were observed following co-exposure of GHSR-1a to both the hydrolysate and ghrelin. Subsequent *in vivo* studies monitored standard chow intake in healthy male and female Sprague-Dawley rats after dosing with the novel casein hydrolysate (CasHyd). Taken together, evidence suggests that the provision of gastro-protected oral delivery of bioactive *in vivo* may aid in the progression of *in vitro* efficacy to *in vivo* functionality. This study thus provides valuable translational data supporting the development of an appetite-enhancing bioactive peptide derived from dairy.



**Keywords:** ghrelin; ghrelin receptor; bioactive peptides; dairy; food intake; appetite; calcium mobilization; cachexia; malnutrition.




***Graphical Abstract****. Graphical synopsis of Chapter 2*

# Introduction

The potential health benefits of the bioactive fragments which exist within the matrix of many food and dietary components have long been known (Nongonierma and FitzGerald 2015). Milk has been identified as one of the richest sources of bioactive fragments and there is a growing body of evidence that these can have positive effects on appetite and metabolism (Phelan and Kerins 2011, Schellekens, Nongonierma et al. 2014, Torres-Fuentes, Schellekens et al. 2015, Nilaweera, Cabrera-Rubio et al. 2017) Many of these bioactives are proven to have various health benefits, with effects spanning the digestive, endocrine, cardiovascular, immune and nervous systems (Fitzgerald and Meisel 2003, Korhonen 2009). Identification and isolation of these bioactives, as well as elucidating their pharmacodynamic parameters are necessary to transfer their potential benefits into functional applications (Korhonen and Pihlanto 2003). The utilisation of dairy-derived bioactives in appetite-related disorders is now becoming increasingly apparent (Nongonierma and FitzGerald 2015, Torres-Fuentes, Schellekens et al. 2015). The ability of a bioactive to enhance satiety and decrease food intake *in vivo* has been shown (Schellekens, Nongonierma et al. 2014). Conversely, recent work has also shown a whey protein isolate to reduce the expression of satiating genes in the hypothalamus and to increase food intake in rodents (Nilaweera, Cabrera-Rubio et al. 2017). However, more translational studies are required to provide insights into the merits and mechanisms of milk-derived bioactives to treat appetite-related disorders.

The endogenous hormone ghrelin, a 28 amino acid peptide is one of the key factors involved in food intake regulation (Kojima, Hosoda et al. 1999). The ghrelin receptor (GHSR-1a) has thus been a therapeutic target for disorders of appetite (Müller, Nogueiras et al. 2015, Howick, Griffin et al. 2017). Particularly, focus has been on individuals with poor appetite secondary to co-morbid conditions such as cardiovascular disease, respiratory disease and cancer, who can suffer an advanced form of 'wasting syndrome' known as cachexia (DeBoer 2008). Ghrelin administration has shown potential as a therapy in cachectic patient cohorts, however therapy is expensive and necessitates intravenous administration (Miki, Maekura et al. 2012, Garcia, Boccia et al. 2015). There have also been a multitude of synthetic ghrelin

ligands developed to date. The most promising of these, anamorelin, has shown robust effects on food intake in humans and is under regulatory review for the treatment of cancer-related cachexia (Temel, Abernethy et al. 2016). In any case, the initiation of pharmaceutical therapy is restricted to patients with co-morbid conditions such as cardiovascular disease, respiratory disease and cancer, who often display cachexia, and would not be routine in mildly reduced appetites, such as those seen in ageing populations. Nevertheless, a ubiquitous decline in ghrelin levels with age is a major contributory factor to appetite reductions and weight loss (Chapman 2004). Poor nutritional status in elderly patients is a complicating factor for chronic diseases and results in prolonged hospital stays, lessened independent living and poorer response to treatment, leading to an overall greater burden on global health infrastructures and lower clinical outcomes (Malafarina, Uriz-Otano et al. 2013). The phenomenon of age-related appetite loss hence represents an important, unmet, clinical need. Given the lack of suitable pharmacotherapy and the growing role of nutraceutical science, we suggest the potential role of a bioactive ghrelin agonist to help delay the onset of a cachectic state due in comorbid illnesses. The pro-active targeting of the ghrelin system with dietary-derived bioactives may precede or supplement pharmacological treatment of clinically significant appetite reductions.

Bioactives that augment the ghrelin system have previously yielded anecdotal evidence of increased appetite, which has since been substantiated by animal and human studies. Rikkunshito (RKT), a long-standing traditional Japanese herb has the ability to function as a ghrelin agonist (Fujitsuka and Uezono 2014). RKT has been shown to reduce weight loss and increase food intake in mouse models of wasting syndrome (Terawaki, Sawada et al. 2014, Tsubouchi, Yanagi et al. 2014), while a retrospective analysis of cancer patients showed increased median survival time in patients receiving concomitant RKT with their treatment (Fujitsuka and Uezono 2014). Chin-shin oolong tea, a popular tea in Taiwan, was empirically perceived to induce hunger, and subsequently was shown to increase food intake in rats (Lo, Chen et al. 2014). *In vitro*, an isolate from Emoghrelin Heshouwu, a Chinese traditional medicine, was shown to activate the GHSR-1a and stimulate GH secretion *in vitro*, supporting a claim for its perceived therapeutic efficacy as an anti-aging supplement (Lo, Chen et al. 2015). Furthermore, an extract from the herbal medicine

*H.procumbens* was shown to act on the GHSR-1a and modulate appetite in an *in vivo* mouse model (Torres-Fuentes, Theeuwes et al. 2014).

There is an impetus to provide dietary-incorporated, scientifically validated interventions for poor appetite at an early point, rather than initiating late-stage pharmaceutical therapy which is often expensive, ineffective and not without side-effects. The proactive use of nutraceutical therapy as a preventative or complementary approach to traditional pharmacotherapy has been recently discussed (Santini, Tenore et al. 2017, Santini and Novellino 2018). There is an impetus for the integration of research disciplines to address the role and mechanism of action of milk protein-derived peptides in health (Nongonierma and FitzGerald 2015). Specifically, investigation of dairy-derived bioactive fragments with the potential to positively affect appetite is warranted in order to inform their clinical usage. Furthermore, bioactive identification, enrichment and incorporation into appropriate delivery systems is required (Howick, Alam et al. 2018). Here, we describe a casein-derived milk hydrolysate (CasHyd) which potently activates the GHSR-1a *in vitro*. In addition, we demonstrate additive and synergistic effects of the hydrolysate with ghrelin. We also investigate the potential of this bioactive peptide to function as an appetite stimulant *in vivo* under different modes of administration. This study thus represents an interesting translational investigation of a novel dairy-derived appetite-stimulating bioactive targeting the GHSR-1a with promising potential for inclusion as a functional food ingredient in population groups with poor appetite who may be at risk of developing malnutrition and cachexia.

# Materials and Methods

## 2.1 Materials

Dairy-derived peptide hydrolysate (CasHyd) was provided by Food for Health Ireland (see section 2.2). Disposable plastic flexible gavage tubes were purchased from Instech Laboratories (Instech Laboratories, Inc. Plymouth Meeting, PA, USA). Standard chow (2018S Teklad Global 18 % Protein Rodent Diet) was procured from Harlan, UK. For encapsulation of bioactive, an aqueous pseudo-latex of EC (Surelease® Type B NF) was sourced from Colorcon Corp., Indianapolis, IN, USA. Microcrystalline cellulose (MCC, Avicel® PH-101 NF Ph. Eur.) was purchased from FMC Corp., Little Island, Cork, Ireland and pharmaceutical grade ethanol 96% (v/v) from Carbon Chemicals Group Ltd., Ringaskiddy, Cork, Ireland. For the $Ca^{2+}$ mobilization assays, fetal bovine serum (3.3%) was obtained from Sigma-Aldrich, Arklow, Wicklow, F7524. Assay buffer was composed of 1x Hanks balanced salt solution, HBSS, Gibco™ 14065049 (Thermo Fisher Scientific™), containing 20 mM HEPES (Sigma-Aldrich, Arklow, Wicklow, H0887). The endogenous agonist, ghrelin (rat), was supplied by Tocris Bioscience, Avonmouth, Bristol, UK (Cat. No. 1465).

## 2.2 Generation of CasHyd

Sodium caseinate (NaCas, Kerry Group Plc, Listowel, Ireland) was suspended at 10 % (w/w) protein basis in water and dispersed under agitation at a pre-defined temperature and duration in an in-line mixer. Protein hydrolysis was carried through addition of food grade enzyme. The pH of hydrolysis was maintained at a constant pH for the duration of hydrolysis by addition of a hydroxide base (Microbio, Fermoy, Ireland). The enzyme was then inactivated by heat treatment through a plate and frame heat exchanger (Unison Engineering Services Ltd., Limerick, Ireland). Large molecular weight material and aggregates were removed from the hydrolysate through membrane separation or clarification steps. The clarified material was then filtered through 1 kDa spiral wound organic membranes (Synder Filtration, California, USA) and the permeate fraction (CasHyd) was dried in a single stage spray dryer (Anhydro F1 Lab Dryer; Copenhagen, Denmark).

## 2.3 $Ca^{2+}$ mobilization assay for peptide GHSR-1a activity

GHSR-1a mediated changes in intracellular $Ca^{2+}$ were recorded on a High-Throughput Cellular Screening System (Molecular Devices Corporation, Sunnyvale, California, USA). $Ca^{2+}$ mobilization assays were performed according to a protocol modified from a previously described method (Pastor-Cavada, Pardo et al. 2016). Stably transfected Human Embryonic Kidney (HEK293A) cells overexpressing GHSR-1a were seeded in sterile 96-well microtiter plates with black-walled and clear-bottomed wells (3904, Costar, Fisher Scientific, Dublin, Ireland) at a density of 2.5 x $10^4$ cells per well. Cells were then kept at 37°C in a humidified atmosphere containing 5 % $CO_2$ overnight. Twenty-four hours before the experiment, media was replaced with serum-free media (1 % non-essential amino acids). On the day of the assays, cells were allowed to incubate with 80 µL of 1xCa5 dye (R8186, Molecular Devices) in assay buffer (1x Hanks balanced salt solution - HBSS, supplemented with 20 mM HEPES buffer). CasHyd was dissolved in assay buffer (1x HBSS supplemented with 20 mM HEPES buffer). Addition of the dissolved compounds (25 µL/well) was performed automatically. Fluorescent readings were taken for 120 seconds at excitation wavelength of 485 nm and emission wavelength of 525 nm. The percentage increase in cytosolic $Ca^{2+}$ was deduced from the difference between maximum and baseline fluorescence and depicted as relative fluorescent units (RFU) normalized to maximum response (reading from 3.3 % fetal bovine serum (FBS)). Background fluorescence from assay buffer alone was subtracted from all readings. The endogenous agonist ghrelin (1465; Tocris) was also used as a positive control of $Ca^{2+}$ influx. Data were analysed using GraphPad Prism software (PRISM 5.0; GraphPAD Software Inc., San Diego, California, USA). Sigmoidal concentration-response curves were generated using nonlinear regression analysis with variable slope.

## 2.4 Calcium imaging

Calcium imaging took place for HEK-GHSR-1a cells seeded on a 12 well plate at $2.0x10^5$ cells/ml two days before the assay according to a previously described method (Pastor-Cavada, Pardo et al. 2016). The day before the assay media was swapped to serum-free. For the assay procedure, all media was removed from cells which were then washed using phosphate buffered saline and incubated for 1 hour at

37ºC with 7uM Fura 2-AM (F1221, Biosciences) in assay buffer. Upon calcium release, the fluorescent excitation maximum of the Fura-2 indicator undergoes a blue shift from 363 nm ($Ca^{2+}$-free) to 335 nm ($Ca^{2+}$-saturated), while the fluorescence emission maximum remains unchanged at 510 nm. Upon excitation at 340 nm and 380 nm respectively, the ratio of the fluorescent intensity emissions at these excitations is correlated to the levels of intracellular calcium. Subsequently, media was replaced with assay buffer without Fura 2-AM. Cells were viewed and a field was selected under brightfield illumination (Olympus BX50WI). Standard digital epifluorescence system (Cell R, Olympus) was used to measure changes in intracellular calcium ($Ca^{2+}$). Light at 340 and 380nm was generated using a Xenon/Mercury arc burner (MT20 illumination system, cell R, Olympus), illuminating the cells and stimulating fura 2 fluorescence. Hydrolysates or the endogenous GHS-R1a receptor agonist ghrelin (SP-GHRL-1, Innovagen) were added and the excitation spectra at 380 nm ($Ca^{2+}$-free) and 340 nm ($Ca^{2+}$-saturated) with fixed emission at 510 nm was recorded.

## 2.5 HPLC characterisation of CasHyd

CasHyd and its parent protein (NaCas) were analysed using size exclusion (SE) high-performance liquid chromatography using a TSK G2000SWXL 7.8 x 300 mm column (Tosoph Corporation, Japan). Analysis was carried out at isocratic conditions for 40 min; the mobile phase was 30 % v/v and 0.1 % v/v TFA in deionised water. Flow rate through the column was 0.5 mL/min. The total injection volume was 20 mL. Absorbance of the eluate was measured at 214 nm. The following molecular weight standards were used for calibration purposes: Tyr-Glu (310 Da), Leu-Trp-Mel-Arg (605 Da), bacitracin (1.4 kDa), aprotinin (6.5 kDa), a-lactalbumin (14.2 kDa) and bovine serum albumin (66 kDa).

## 2.6 Cell culture, *in vitro* transfection and lentiviral transduction

Hek293A and SHSY5Y cells were maintained in culture in high glucose Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% heat inactivated fetal bovine serum and 1% non-essential amino acids in an atmosphere of 95% air and 5% $CO_2$ at 37 °C. Hek293A cells were transfected using lipofectamine LTX plus reagent (Invitrogen) with a GHS-R1a-EGFP construct (EX-X0963-M03,

Genecopeia) according to manufacturer's instructions. Cells stably expressing the GHS-R1a receptor with the C-terminal-EGFP fusion protein, were selected using geneticin (G418, Merck) as a selection antibiotic. Cell populations with the highest fluorescence were selected using flow assisted cell sorting (FACS). In addition, SHSY5Y cells were transduced to express the GHS-R1a receptor using a 3rd generation packaging, gene delivery and viral vector production system developed by Naldini and colleagues (Naldini, Blomer et al. 1996, Vigna and Naldini 2000, Follenzi and Naldini 2002, Follenzi and Naldini 2002). HIV-based lentivector (LV) particles expressing the GHS-R1a from a spleen focus-forming virus (SFFV) promoter in conjunction with an EmGFP sequence expressed as a separate protein after an internal ribosome entry site (IRES) were generated. Briefly, the GHS-R1a sequence was cloned into a HIV-based, replication deficient, lentiviral expression plasmid, pHR-SIN-BX-IRES-EmGFP (kind gift of Adrian Thrasher, Institute of Child Health, London, United Kingdom), modified to exclude the shRNA U6 promoter. The GHS-R1a gene was amplified, gel isolated using the Qiagen Gel Extraction Kit (#28706) and ligated into the lentiviral vector using BamHI and XhoI restriction sites, generating pHR-GHS-R1a-IRES-EmGFP. Lentivector (LV) GHS-R1a expressing particles, pseudotyped with the vesicular stomatitis virus G [VSV-G] were produced using 293T-17 cell following transient cotransfection of the cloned expression constructs, pHR-GHS-R1a-IRES-EmGFP, the packaging construct, pCMVΔR8.91 and the envelope construct, pMD.G –VSVG. SHSY-5Y cells were transduced with the GHS-R1a expressing lentiviral vectors diluted in transduction media, consisting of DMEM with 2% heat-inactivated FBS, 1% NEAA and an additional 8μg/ml polybrene® (Sigma; #H9268). Fluorescence was monitored using flow cytometry as indicator of receptor expression.

## 2.7 Cumulative Food intake studies

Male and female Sprague-Dawley (SD) rats were purchased from Envigo, UK. Rats were 7 to 8 weeks-old when received at the facility. Animals were group-housed (4 rats per cage) in standard holding cages with controlled light-dark cycle (12-h light; lights on at 7:00 a.m.) and in a temperature- (21 ± 1°C) and humidity-controlled (55 ± 10 %) environment. Water and standard lab chow (2018S Teklad Global 18 % Protein

Rodent Diet, Envigo, UK) were available *ad libitum*. All experiments were in full accordance with the European Community Council directive (86/609/EEC) and approved by the Animal Experimentation Ethics Committee of University College Cork (B100/3774). Animals were habituated to experimental conditions for a week prior to experiments taking place. On experimental day, animals were administered their respective treatment at the onset of the light phase and then placed in individual cages for duration of food intake monitoring. Food intake was then recorded by weighing the chow at defined intervals. For the gastro-protected pellets, animals were food restricted for a period of 4 hrs before a pre-weighed quantity of chow was added to the cages. The dosing system for pellets consisted of a flexible PVC gavage tube which was filled with a pre-weighed quantity of blank or active pellets. After insertion of the dosing tube a guidewire was used to administer the dose of pellets directly into the stomach.

## 2.8 Pellet preparation by extrusion-spheronisation

Requisite quantities of CasHyd and MCC were combined in a ratio of 33:67 and manually blended for 1 minute. A Kenwood planetary mixer (KM005, Kenwood Ltd., Hampshire, UK) was then used to further dry blend the mixture for 5 minutes at a minimum agitation setting. The dry powder blend was gradually wetted by adding deionized $H_2O$, under constant agitation by the planetary mixer. The granulation end-point was achieved upon addition of a cumulative amount of deionized $H_2O$ equivalent to 45 % (w/w) of the dry powder blend. The granulate was immediately extruded at an extrusion speed of 17 – 19 rpm using a sieve extruder (Caleva® Extruder 20, Caleva Process Solutions, Sturminster Newton, Dorset, UK). Screen thickness and aperture diameter were both 1 mm. The extrudate was then placed into a Caleva® Spheroniser 250 for 90 seconds at 1500 rpm (Caleva Process Solutions, Sturminster Newton, Dorset, UK). Pellets were collected and dried using high flow air in a microfluid bed system (Vector Corp., Marion, IA, USA) at 40 °C for 20 minutes before coating took place.

## 2.9 Pellet film coating

Film coating was performed in a laboratory scale microfluid bed system in bottom-spray mode. Nozzle air was set to 16-17 psig and airflow was 310 – 335 L/minute. Coating solution, an 11% (w/w) aqueous pseudo-latex of EC (Surelease® Type B), was fed at a constant rate (1.0 gram/minute). Prior to coating, the Surelease polymer was allowed to homogenise for 30 minutes under constant agitation. Uncoated pellets were charged to the coating vessel and pre-heated for 10 minutes with an inlet air temperature of 80 °C, such that the sufficient drying could be obtained of the coating polymer. This was achieved at an outlet air temperature of ~ 50 °C. The amount of coating polymer required for film coating was calculated as a theoretical % weight gain based on a pre-based on the weight of uncoated pellets at the start of coating. The microfluid bed coating system was constantly monitored to ensure that appropriate air flow and drying was maintained in the coating chamber.

## 2.10 pH susceptibility tests

CasHyd was dissolved in deionized $H_2O$ and acidified with HCl to the requisite pH (pH 1, 3, 5 and untreated), using a pHenomenal® 1000L pH meter and electrode. Acidified CasHyd solutions were incubated for 30 minutes under gentle agitation. 50 µL of each sample was removed and added to 950 µL of $Ca^{2+}$ assay buffer and pH checked to confirm that acidity was neutralized before samples were added to cells.

## 2.11 *In vitro* dissolution studies

Dissolution testing (USP Type 1) was performed, using a basket-type dissolution apparatus (DISTEK, Inc., Model 2100C, North Brunswick, NJ, USA). Simulated gastric fluid sine pepsin (SGFsp) (pH 1.2, 500mls) was used as dissolution media. Dissolution bath temperature was kept at 37 °C and 50 rpm agitation speed. Sampling was conducted at various timepoints. After each sample an equal volume of dissolution medium was added to the dissolution vessel to maintain volume at 500ml.

## 2.12 Peptide quantification assay

The bicinchoninic acid (BCA) assay was performed using a BCA assay kit (Thermo Fisher Scientific™ Pierce™ BCA Protein Assay, Catalog Number 23225) according to a well-established method. A 2 mg/ml stock solution of CasHyd in SGFsp was used to prepare a series of dilutions for preparation of a standard curve (2, 1, 0.5, 0.25, 0.125, and 0.0625 mg/mL, respectively). 25 µL of each sample obtained during dissolution testing, and standards were plated on a 96-well plate. After the dissolution experiment was completed, remaining pellets were removed, crushed, and quantified as above in order to confirm all peptide was released from the formulation. Working reagent was prepared by mixing BCA assay Reagent A with BCA assay Reagent B in a ratio of 50:1. The working reagent (200 µL) was then transferred to each well. The plates were then covered and incubated at 37 °C for 30 minutes. Spectrophotometric analysis was performed at 562 nm and quantity of peptide in each sample was quantified from standard curves and expressed as a % of total peptide in the pellets.

## 2.13 Data Analysis

Data were analysed and graphs generated using both GraphPad Prism software and Microsoft Excel software. For *in vitro* cell screening and dissolution work, all means were calculated from the results of at least three independent experiments carried out in triplicate. For the *in vitro* calcium mobilization assays, standard error of the mean (SEM) is depicted, the dissolution result reports standard deviation (SD). For *in vivo* food intake, measurements between groups were analysed using a one-way, repeated measures ANOVA followed by estimation of parameters. If data was non-spherical as determined by Mauchly's test for sphericity, a Huynh-Feldt correction was applied for data analysis. Graphs are expressed as mean ± SEM. Statistical significance was indicated as follows: * indicates $p<0.05$; ** indicates $p<0.01$ & *** indicates $p<0.001$.

# Results

## 2.14 Activity of CasHyd on ghrelin receptor overexpressing cell line.

The activity of the casein-derived hydrolysate, CasHyd, on the GHSR-1a was analysed using an intracellular $Ca^{2+}$ mobilization assay, as a measure of downstream GHSR-1a signalling activation (Schellekens, van Oeffelen et al. 2013), in HEK293A cells stably expressing the GHSR-1a tagged with an enhanced green fluorescent protein (GHSR-1a-EGFP) (Figure 2.1).

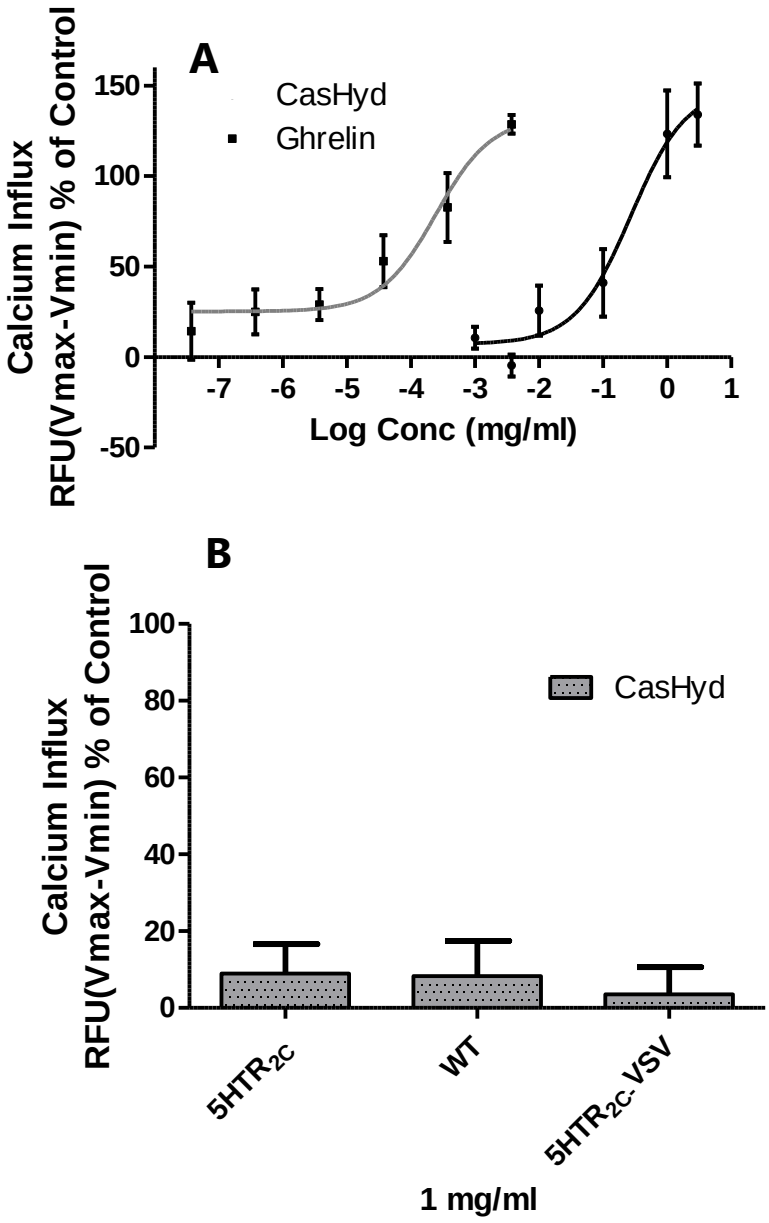



***Figure 2.1. A Concentration response curve of novel casein-derived hydrolysate****. Concentration response curve for the casein-derived hydrolysate, CasHyd measured in GHSR-1a over-expressed in HEK293A cells.* ***B*** *Activity of CasHyd in wild-type (HEK293A-WT) cells, $5HT_{2C}$ receptor (HEK293A-$5HTR_{2C}$) and a fully edited form of $5HTR_{2C}$ (HEK293A-$5HTR_{2C}$ -VSV) expressing cells. Intracellular $Ca^{2+}$ increase was depicted as a percentage of maximal $Ca^{2+}$influx in relative fluorescence units (RFU) as elicited by control (3.3% FBS). Graph represents mean ± SEM of at least three independent experiments performed in triplicate.*

***Table 2.1.*** *Activity of CasHyd and Ghrelin on GHSR-1a over-expressing HEK293A cells*

| Compound | $EC_{50}$ | $E_{max}$[1] |
|---|---|---|
| Ghrelin | 0.25 µg/ml | 132.5% |
| CasHyd | 0.27 mg/ml | 148.9% |

[1] *Intracellular $Ca^{2+}$ increase reported as a percentage of maximal $Ca^{2+}$influx in relative fluorescence unit (RFU) as elicited by control (3.3% FBS).*

CasHyd stimulated calcium mobilization in cells expressing GHSR-1a in a concentration-dependent manner, with the $EC_{50}$ = 0.27 mg/ml and efficacy ($E_{max}$) reaching 148.9 %. The potency of CasHyd was 1000-fold lower than that for the endogenous receptor ligand, ghrelin ($EC_{50}$ = 0.25 µg/ml, $E_{max}$ = 132.5%). Considering CasHyd is a mixture of different peptides not all of which are likely to elicit bioactivity, the activation found here on the GHSR-1a indicates promising ability to modulate the receptor. Efficacy of CasHyd was normalized to the maximal response of the positive control (3.3% FBS, $E_{max}$ = 100%). Critically, the concentration response curve shows that the hydrolysate has GHSR-1a agonist activity, while no $Ca^{2+}$ influx was observed in wild-type HEK293A cells (HEK293A-WT) not expressing the GHSR-1a. Furthermore, no activity was observed in $5HT_{2C}$ (HEK293A-$5HTR_{2C}$) receptor-expressing cell line, nor in the edited from of the $5HTR_{2C}$, (HEK293A-$5HTR_{2C}$ -VSV), compared to treatment with control (FBS), which gives maximal intracellular $Ca^{2+}$ mobilization in all tested cell lines. Food intake and adiposity are altered *in vivo* when the 5-$HT_{2C}$ receptor RNA is fully edited, suggesting a potential role for 5-$HT_{2C}$ editing in eating disorders (Schellekens, Clarke et al. 2012). Together, these results show to our knowledge for the first time, the promising potential of the novel CasHyd to specifically modulate the GHSR-1a.

## 2.15 Calcium imaging on ghrelin receptor overexpressing cell line

Next, the GHS-R1a mediated calcium response of the HEK-GHSR-1a cells to ligand exposure was investigated using calcium imaging. Following addition of 500nM ghrelin an increased fluorescence peak is observed indicating calcium influx due to treatment. This calcium influx is also observed upon the acute addition of

CasHyd (1.5mg/ml), but to a lesser extent than was seen upon ghrelin addition (Figure 2.2A). Calcium influx is evident through the shift in fluorescence from pink to blue (Figure 2.2B), indicating calcium release from intracellular stores resulting in an excitation shift from 340nm to 380nm. This further corroborates the calcium mobilization results obtained.

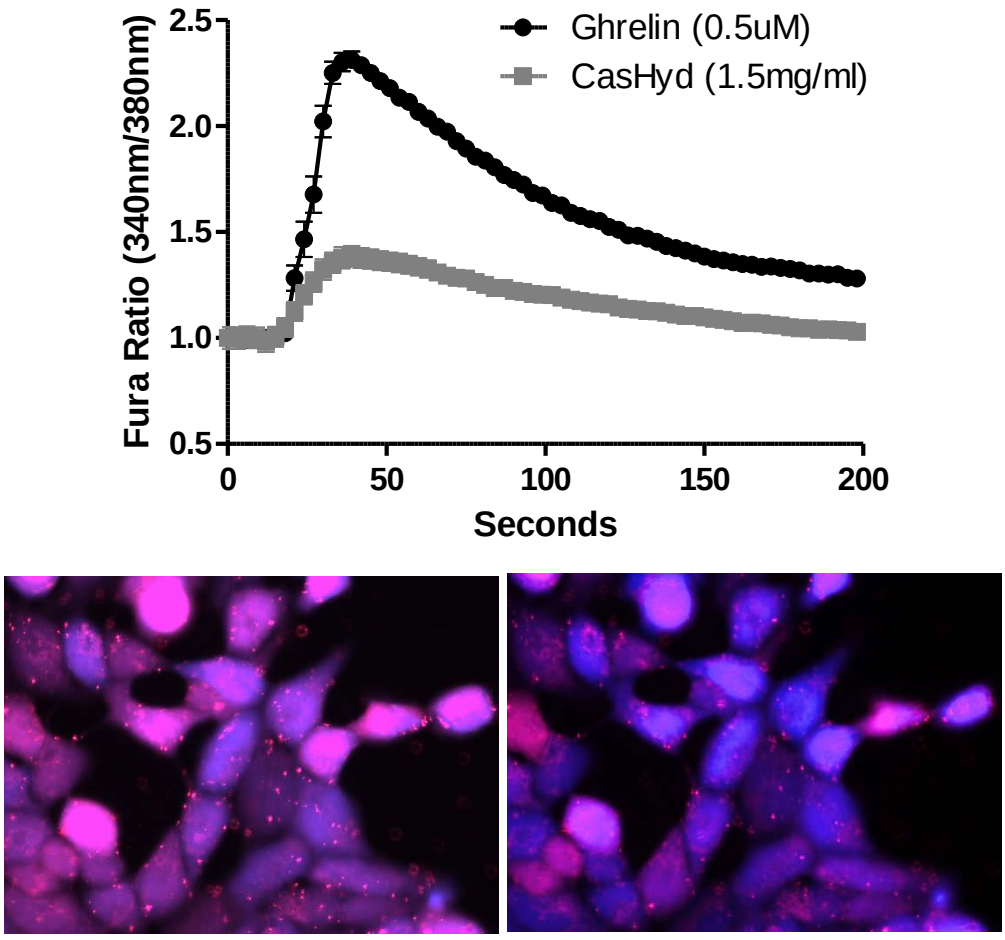



***Figure 2.2. Specific activation of heterologously expressed GHSR-1a in HEK293A cell-line.*** *Calcium imaging of HEK cells (60x magnification) heterologously expressing the GHS-R1a. Cells were seeded for 48 hours into wells at a density of $2.0x10^5$ cells/ml, and loaded for 1 hr with the UV-excitable fluorescent calcium indicator, Fura-2AM, and the 340nm/380nm ratio is recorded after addition of 500nM ghrelin or 1.5 mg/ml CasHyd. Traces represents the average of three independent experiments, dotted lines indicative of SEM.*

## 2.16 HPLC characterization of CasHyd

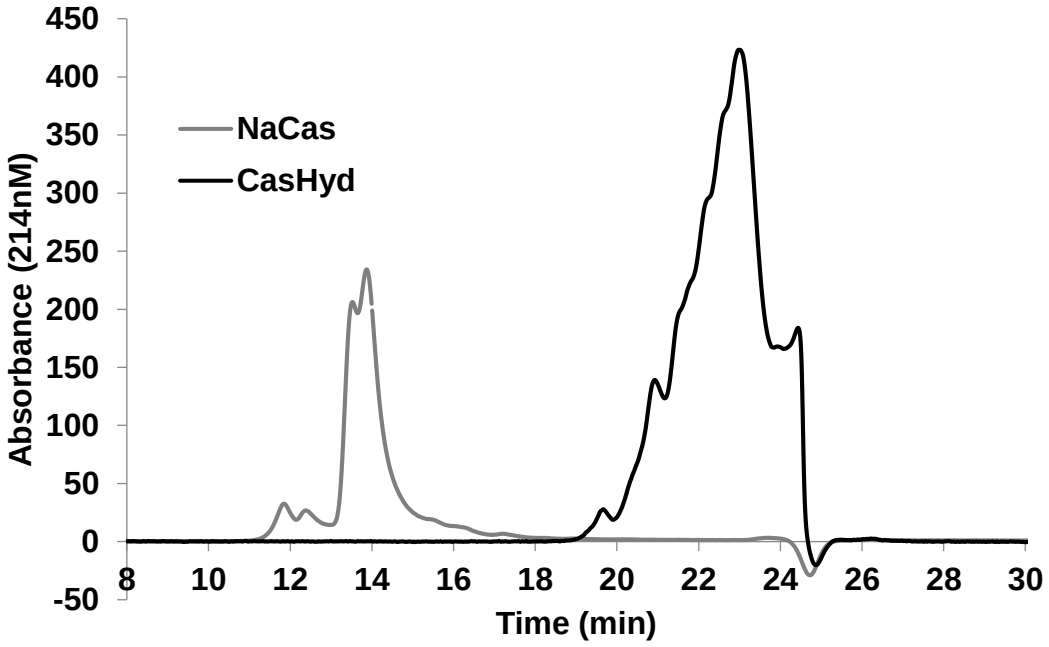



***Figure 2.3. Size exclusion HPLC for CasHyd fraction compared with parent casein protein.*** *Molecular weight distribution of CasHyd and parent protein, Sodium Caseinate (NaCas), expressed as absorbance over time on HPLC chromatogram.*

Size exclusion chromatography carried out on the whole unhydrolysed protein, Sodium Caseinate (NaCas) versus the hydrolysate, CasHyd, shows no overlap in the molecular weight distribution after enzymatic hydrolysis. The unhydrolysed parent protein, NaCas, showed 85.9% of total proteins to be >25kDa molecular weight, whereas 86.0% of CasHyd is below 1kDa in size. This shows the extent of hydrolysis which takes place yielding a mixture of vastly different peptide fractions to the parent casein protein. In addition, the high level of hydrolysis of CasHyd yielding a majority of peptides <1kDa, is likely to contribute to the observed bioactivity of GHSR-1a modulation.

***Table 2.2.*** *Molecular Weight distribution of NaCas and CasHyd.*

| **Molecular Weight** | **Retention Time (min)** | **NaCas (% Area)** | **CasHyd (% Area)** |
|---|---|---|---|
| >25 kDa | <14.66 | 85.9 | 0.0 |
| 25 kDa – 10kDa | 14.66-17.26 | 13.1 | 0.0 |
| 10kDa – 5 kDa | 17.26-19 | 1.0 | 0.0 |
| 5kDa – 1kDa | 19-21.35 | 0.0 | 14.0 |
| <1kDa | >21.35 | 0.0 | 86.0 |

## 2.17 GHSR-1a activation by CasHyd in neuronal cells *in vitro*.

Next, the activity of CasHyd was assessed in the neuroblastoma cell line, SHSY-5Y, engineered to overexpress the GHS-R1a as a native receptor (no fluorescent tag) using lentiviral vectors. A calcium mobilization response following exposure to the endogenous ligand, ghrelin, as well as the dairy-derived hydrolysate, CasHyd, was observed in both engineered cell lines. We also demonstrate hydrolysate-mediated calcium mobilization in neuronal cells endogenously expressing the GHS-R1a receptor (Figure 2.4).

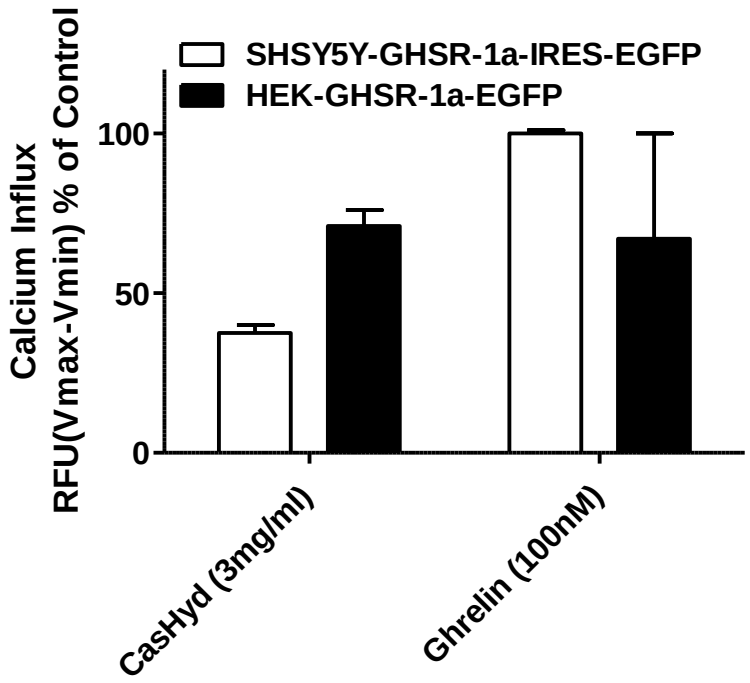



***Figure 2.4. Specific activation of tagged and untagged GHS-R1a receptor in neuronal cells.*** *Both CasHyd (3mg/ml) and ghrelin (100nM) elicited a GHS-R1a mediated calcium influx in the neuronal-like cell line (SHSY-5Y-GHSR-1a_-IRES-EGFP) and HEK-GHSR-1a-EGFP cells generated to express the receptor using lentiviral vectors. Graphs represent the mean +/− SEM of a representative experiment with each concentration point performed in triplicate. Intracellular calcium increase was depicted as a percentage of maximal calcium increase as elicited by control (100nM ghrelin)(IRES – Internal ribosome entry sites, EGFP – enhanced green fluorescent protein) .*

## 2.18 Additive and synergistic effects of the novel dairy-derived hydrolysate on ghrelin-mediated GHSR-1a activation.

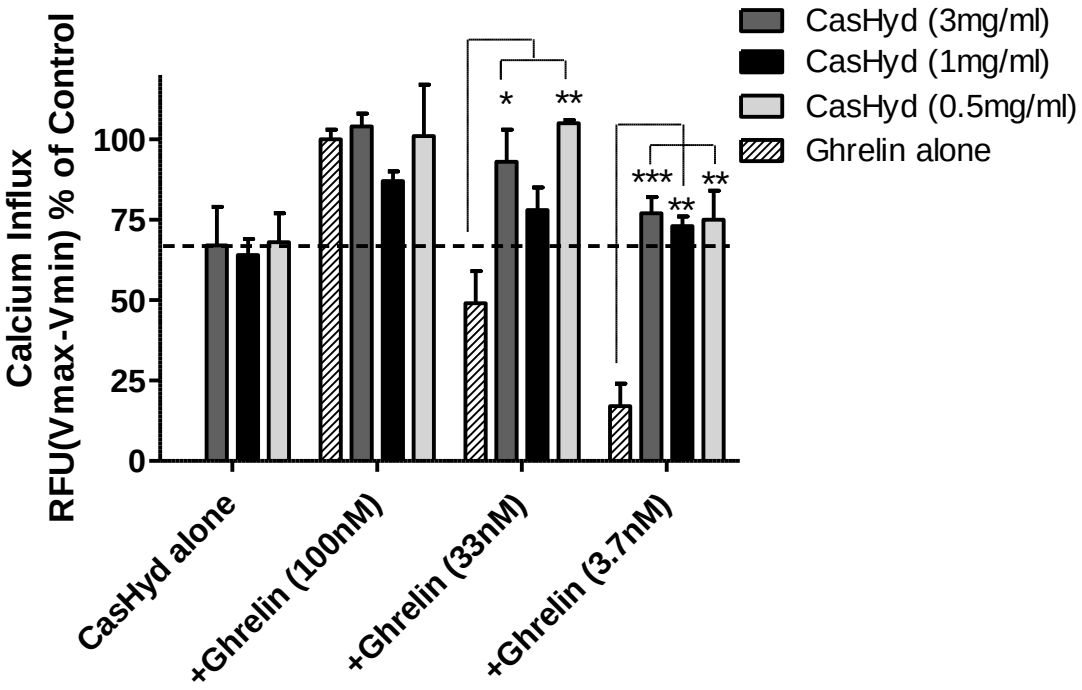



***Figure 2.5. Additive GHS-R1a activation following co-treatment of ghrelin and CasHyd****. Additive calcium mobilization is observed following co-treatment of HEK cells stably expressing the GHS-R1a with 33nM of ghrelin and CasHyd. Graphs represent the mean +/− SEM of a representative experiment of three independent with each concentration point performed in triplicate. Intracellular calcium increase was depicted as a percentage of maximal calcium increase as elicited by control (100nM ghrelin). Statistically significant differences are calculated using a one-way analysis of variance (ANOVA) followed by Bonferroni post-hoc test for multiple comparisons and depicted as ***p<0.001, **p<0.01 and *p<0.05.*

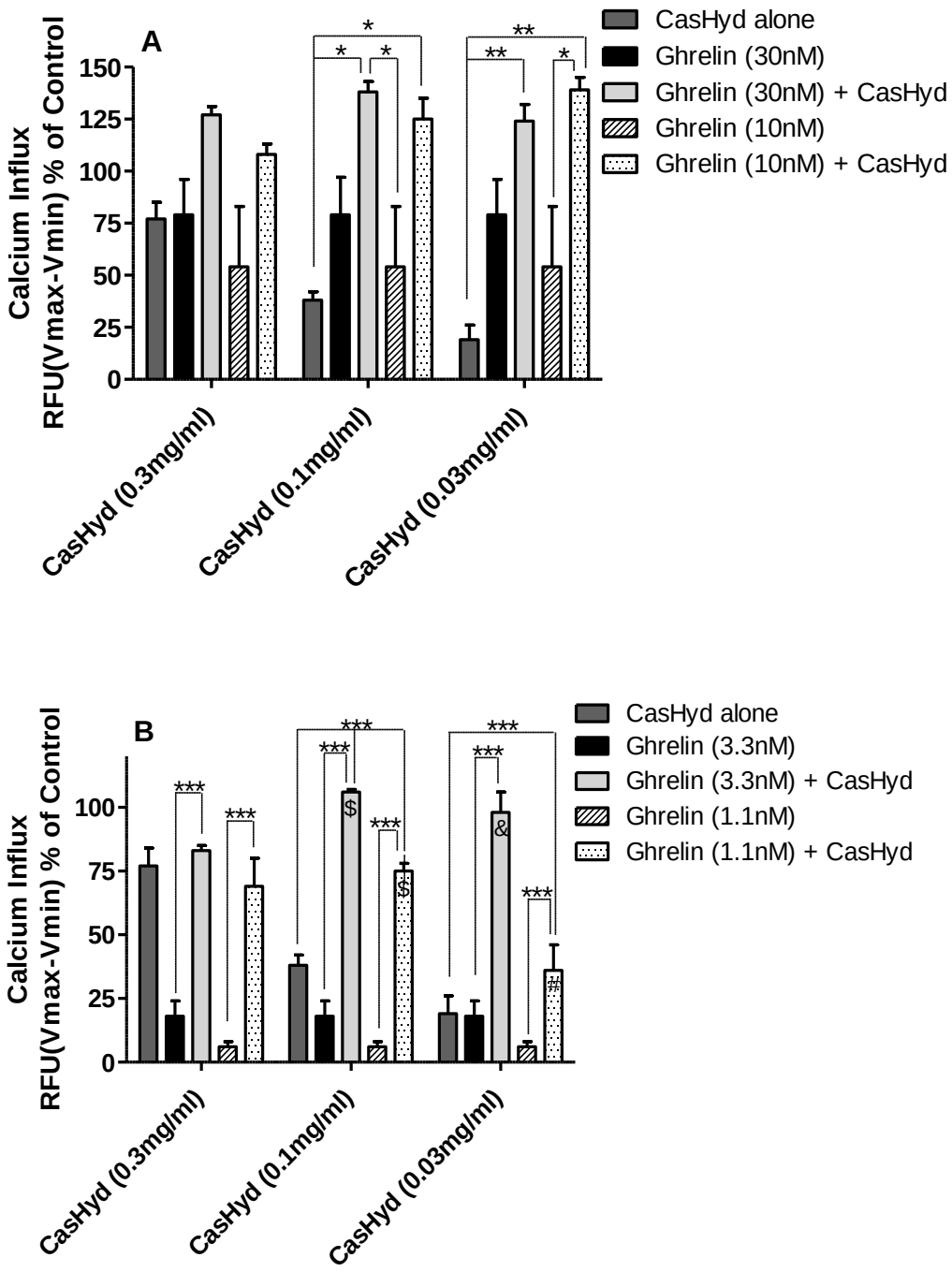



***Figure 2.6. Additive and synergistic effects of GHS-R1a activation following co-treatment of ghrelin and CasHyd.*** *Additive and synergistic effects are observed following co-treatment of HEK cells stably expressing the GHS-R1a with hydrolysate and 30nM or 10nM ghrelin (A) or hydrolysate and 3.3nM or 1.1nM ghrelin (B). Intracellular calcium increase was depicted as a percentage of maximal calcium increase as elicited by control (100nM ghrelin). Graphs represent the mean +/− SEM of a representative experiment of three independent with each concentration point performed in triplicate. Statistically significant differences of combination treatment*

*indicating an additive effect are calculated using a one-way analysis of variance (ANOVA) followed by Bonferroni post-hoc test for multiple comparisons and depicted as ***p<0.001, **p<0.01 and *p<0.05. Statistically significant differences of a synergistic effect are depicted as; $ p<0.001, & p<0.01 and # p<0.05.*

Potential additive or synergistic effects between ghrelin and the novel dairy-derived hydrolysate on GHS-R1a activation were investigated. Cells stably expressing the GHSR1a were exposed to different concentrations of ghrelin (100nM-3.7nM) and CasHyd (3-0.5mg/ml). Increases in intracellular calcium could be observed following all concentrations of hydrolysate and a dose dependent calcium influx for ghrelin (Figure 2.5). However, no synergistic effects were observed. A small additive effect was observed for cells treated with a suboptimal concentration of ghrelin (33nM) and all three concentrations of CasHyd but this did not reach statistical significance. However, when analysing the effect on calcium mobilization using lower concentrations of CasHyd (0.1mg/ml and 0.03 mg/ml) clear additive effects could be observed with 30nM and 10nM ghrelin (Figure 2.6A). In addition, additive calcium mobilization was observed following co-treatment of 0.3 mg/ml CasHyd and 3.3nM or 1.1 nM ghrelin (Figure 2.6B). Moreover, synergistic effects were observed when cells were co-treated with the two lowest concentrations of CasHyd (0.1mg/ml or 0.03mg/ml) and ghrelin (3.3nM and 1.1nM).

## 2.19 Cumulative food intake studies after intraperitoneal injection of peptide solution

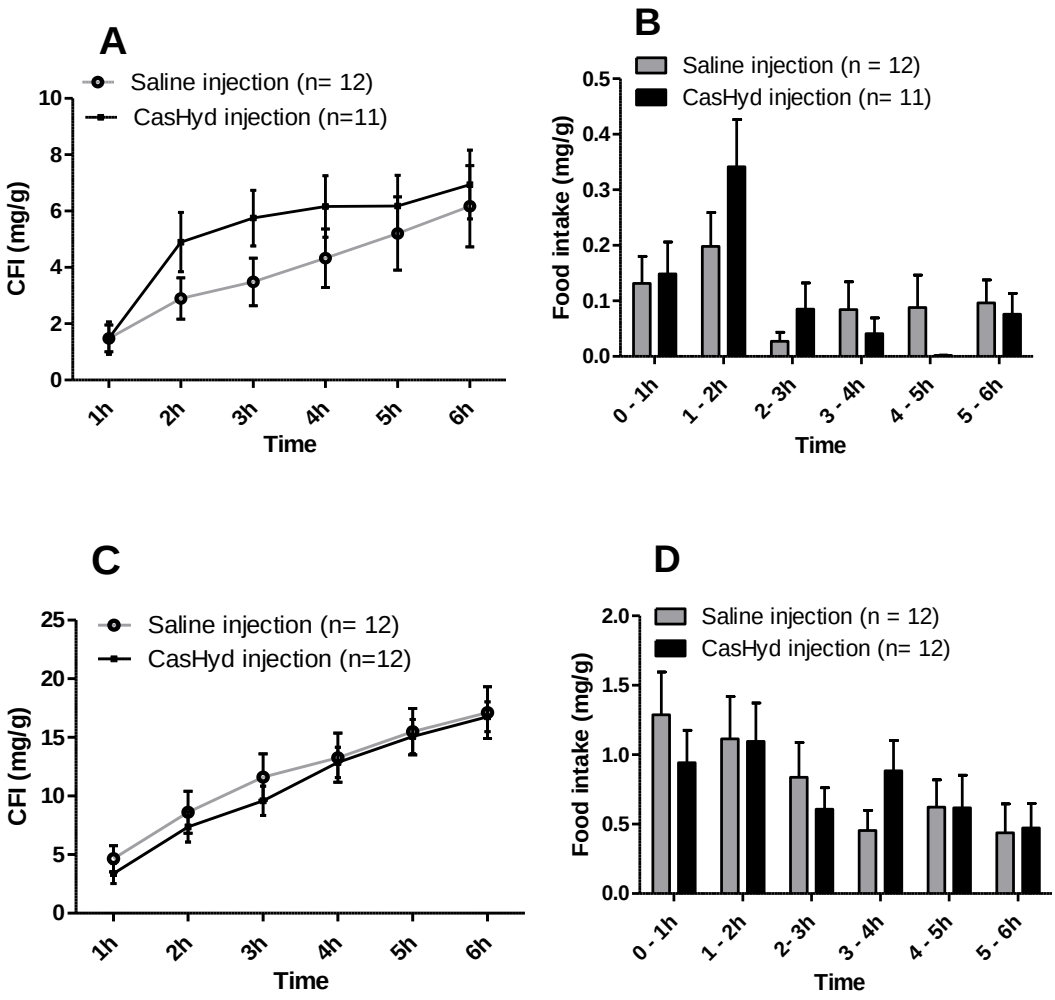



***Figure 2.7. Cumulative food intake following intraperitoneal administration of dairy derived peptide hydrolysate.*** *Cumulative food intake (CFI) (regular chow) intake in male (A) and female (C) sprague-dawley rats was determined following intraperitoneal injection with 50mg kg$^{-1}$ body weight of CasHyd over 6 hours. The food intake per time bin is also illustrated for males (C) and females (D). Data presented as mean ± SEM.*

In food intake studies following an intraperitoneal injection of CasHyd 50mg kg$^{-1}$ in 0.9% saline, or control, there were no significant differences noted in the amount of food consumed between groups, normalized to body weight. Examination of individual time bins yielded no overall differences at any timepoint. Hence, we conclude that CasHyd is not effective as an appetite stimulant with this mode of delivery.

## 2.20 Cumulative Food Intake studies after oral administration of peptide solution

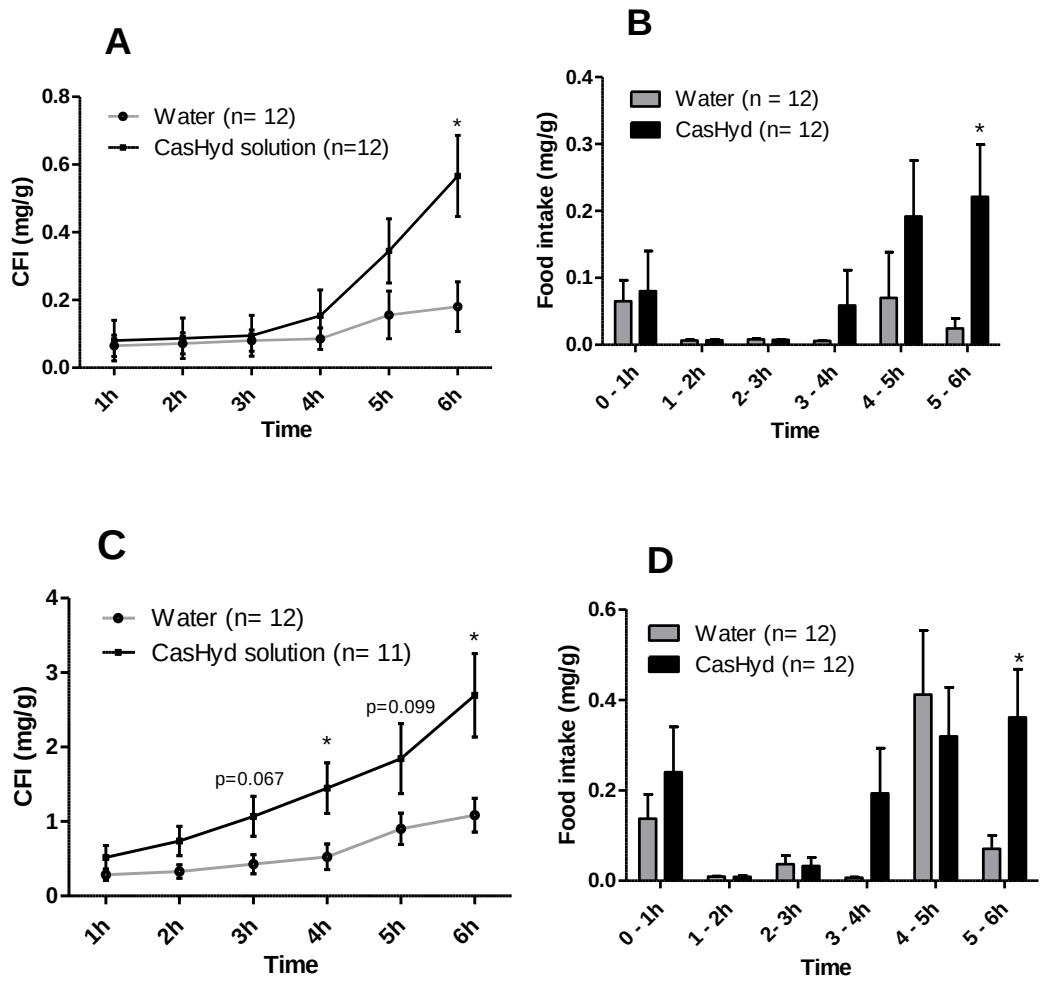



***Figure 2.8. Cumulative food intake following oral administration of unencapsulated dairy protein-derived hydrolysates.*** *Food (regular chow) intake in male (A) and female (B) sprague-dawley rats was determined following oral gavage with 50mg kg$^{-1}$ body weight of CasHyd over 6 hours. Cumulative food intake (CFI) was determined at regular intervals after oral gavage. The food intake per time bin is also illustrated (B and D). Graphs represents the mean ± SEM. Statistical significance was determined using repeated measures ANOVA and estimation of parameters for food intake. Pairwise comparisons were carried out using Tukey's post-hoc test, while independent samples t-test was used for each individual timebin; statistical significance is depicted as **p < 0.01 and *p < 0.05.*

In cumulative food intake (CFI) studies following an oral gavage of a 50 mg kg$^{-1}$ dose of peptide solution, there were significant increases noted in the amount of food consumed relative to control in both males and females, normalized to body weight (Males, p=0.013, Females p = 0.021; Huyn-Feldt sphericity correction).

Pairwise comparisons also reveal trends at 3 and 5 hours post dose, and a significant increase at 4 hours compared to control for the female cohort. The most significant change in food intake was in the 5-6 hour time bin for both males and females (B, D). The GHSR-1a is located on vagal nerve terminals in the gastrointestinal tract, throughout the small and large intestine (Howick, Alam et al. 2018), potentially explaining the increased efficacy of the oral route versus IP via potential local GHSR-1a stimulation.

## 2.21 pH susceptibility of CasHyd

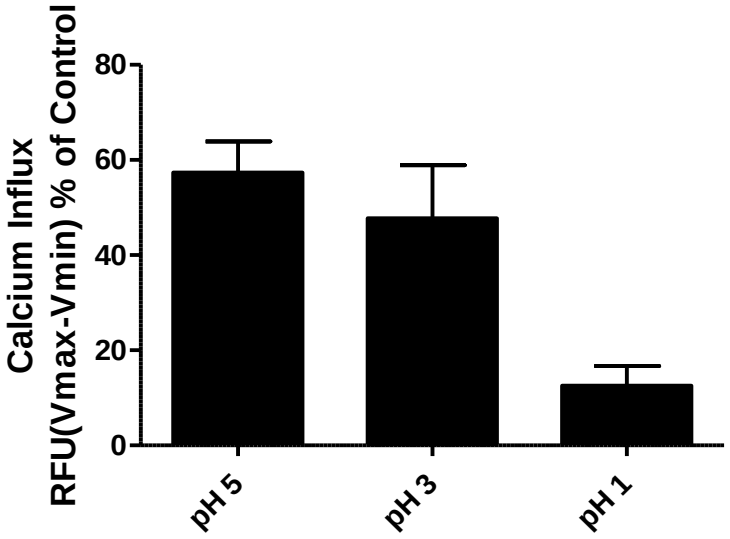



***Figure 2.9. GHSR-1a agonist, CasHyd, displays pH dependent activity***. *Reduction in hydrolysate-mediated GHSR-1a activation following exposure to acidic pH confirms the need for an oral delivery mechanism. Graph represents three independent experiments carried out in at least triplicate (Control = CasHyd not exposed to acidic pH, RFU = Relative Fluorescence Units).*

CasHyd was exposed to varying degrees of acidic pH for a time representative of minimum gastric residence time in the fasted state (minimum 30 minutes (Tuleu, Andrieux et al. 1999)Howick, 2018 #1307). A pH-dependent loss in peptide activity is observed for CasHyd, confirming the need to develop a gastro-resistant formulation to minimize exposure to gastric acid before progression to further *in vivo* efficacy studies (Figure 2.9).

## 2.22 Delivery system (pellet) characterization work

Since CasHyd is susceptible to acidic pH, the peptide was incorporated into a gastro-protected delivery system (coated pellets) in order to minimize exposure to the stomach *in vivo.* Simulated release profile assessment of CasHyd from the formulation was carried out *in vitro* in order to assess whether the coating applied to the pellets was able to delay release. USP Type 1 (Basket) dissolution studies were carried out in gastric conditions (simulated gastric fluid, SGFsp, pH 1.2) in order to confirm a delayed release of peptide from the pellets. Pellets displayed a delayed release of peptide load over 60 minutes, confirming the functionality of the delivery system (Figure 2.10).

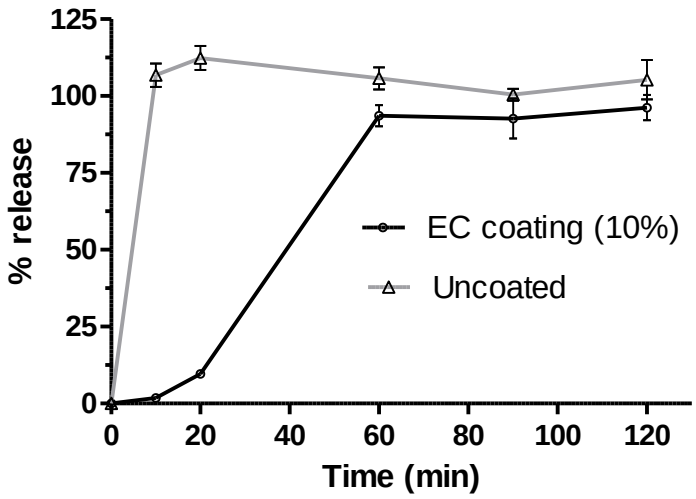



***Figure 2.10. Dissolution study of gastro-protected CasHyd pellets.*** *USP Type 1 (Basket) dissolution studies (50 rpm, 37.5 °C) showed gastro-protected release in simulated conditions (Simulated Gastric Fluid sine pepsin (SGFsp) pH 1.2).*

## 2.23 GHSR-1a activity of peptide post-encapsulation

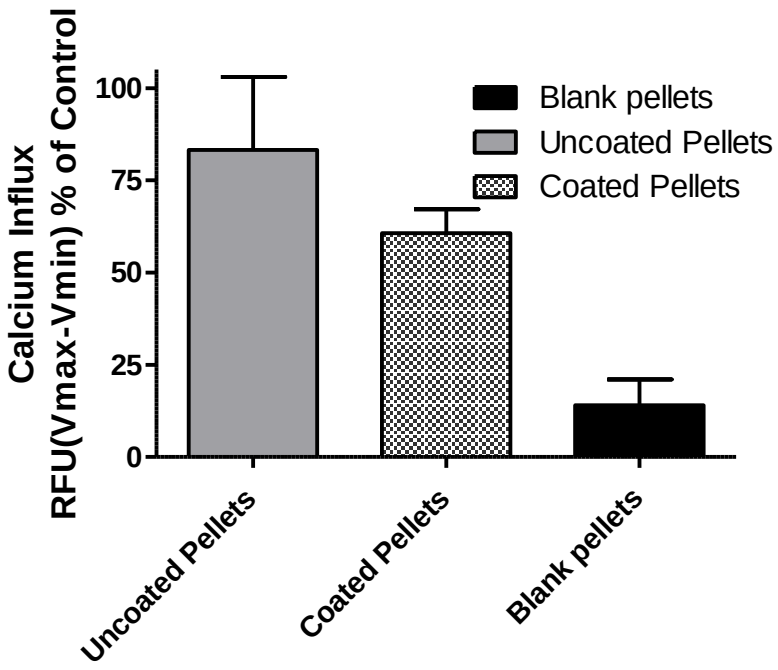



***Figure 2.11. CasHyd retains bioactive functionality after encapsulation.*** *Activity of CasHyd after encapsulation was determined relative to activity of non-encapsulated CasHyd in GHSR-1a- expressing cells. Activity was quantified as being 75 % for uncoated pellets and 60% for coated pellets (representative of three independent experiments carried out in triplicate).*

Due to the likely fragile nature of the peptide hydrolysate (Howick, Alam et al. 2018), we quantified the impact of the encapsulation processing conditions on bioactivity. Activity of CasHyd in the encapsulated pellets was determined relative to activity of non-encapsulated CasHyd peptide in the GHSR-1a overexpressing cells, as described above. Activity was quantified as being 75 % for uncoated pellets and 60% for coated pellets.

## 2.24 Cumulative food intake studies after oral administration of peptide encapsulated in gastro-protective pellets

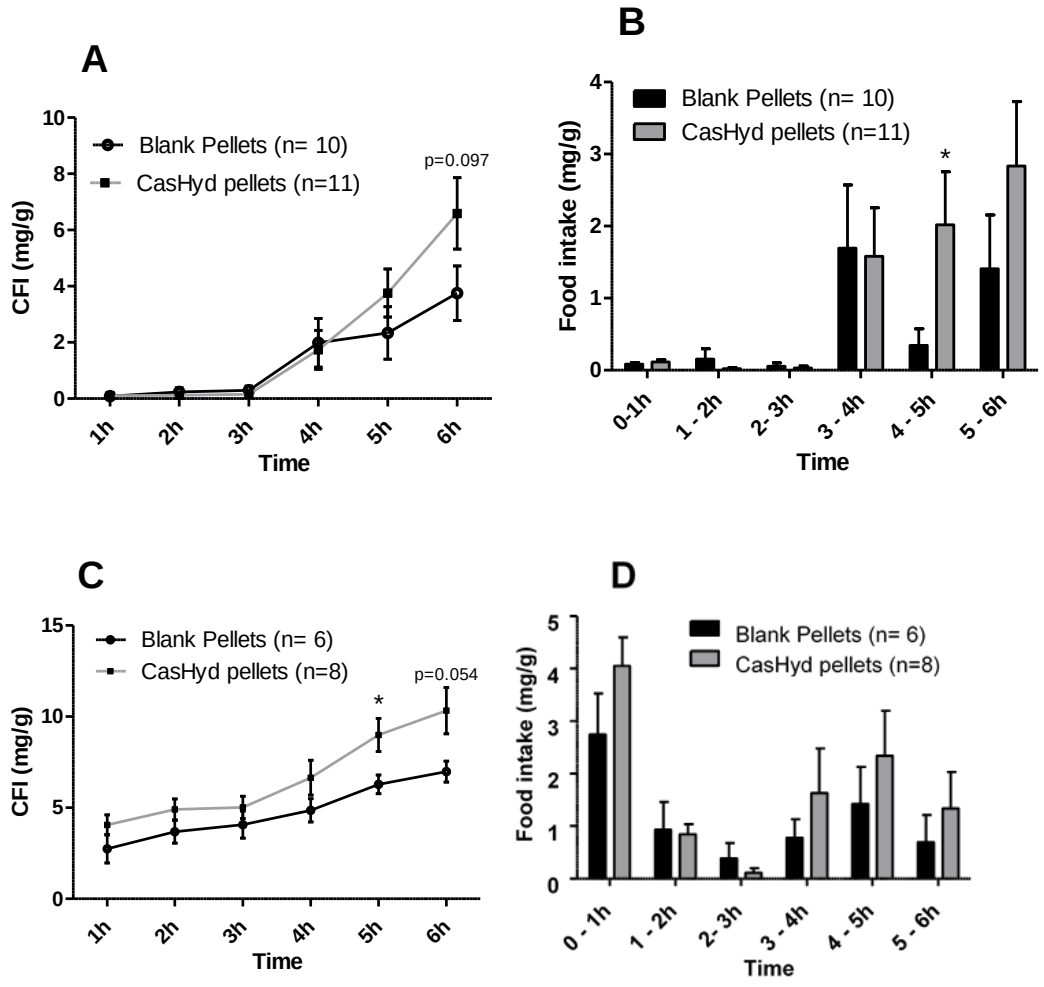



***Figure 2.12. Cumulative food intake following oral administration of gastro-protective pellets containing casein-derived hydrolysate.*** *Food (regular chow) intake in male (top) and female (bottom) Sprague-Dawley rats was determined following oral gavage with 35mg kg$^{-1}$ dose of encapsulated CasHyd over 6 hours. Cumulative food intake (CFI) was determined at regular intervals, beginning 4 hours after oral gavage of the coated pellets containing CasHyd, or an equivalent weight of blank pellets. The food intake per time bin is also illustrated. Graphs represents the mean ± SEM. Statistical significance was determined using repeated measures ANOVA and estimation of parameters for food intake. Pairwise comparisons were carried out using Tukey's post-hoc test, while independent samples t-test was used for each individual timebin; statistical significance depicted is notated as **p < 0.01 and *p < 0.05.*

In food intake studies following an oral gavage of casein hydrolysate encapsulated in a coated pellet formulation vs. a blank pellet formulation, there were

no overall significant increases noted in the amount of food intake for males or females, however a trend towards an overall increase is noted at the 6 hour timepoint for both. A significant increase in amount of food consumed was observed in the 4-5hour time bin for the male cohort also. However, the orexigenic effect seen after oral dosing of the unencapsulated peptide is not confirmed here.

# Discussion

Ghrelin and the growth hormone secretagogue receptor, or GHSR-1a, play an important role in energy balance and appetite regulation (Schellekens, Dinan et al. 2010, Howick, Griffin et al. 2017). Many studies have reported potent appetite-stimulating effects of both peripheral and central administration of ghrelin (Tschop, Smiley et al. 2000, Wren, Seal et al. 2001). Furthermore, natural analogues of ghrelin have provided anecdotal and, more recently, experimental evidence of a positive effect on appetite and energy balance in susceptible population groups (Fujitsuka and Uezono 2014). Hydrolysates of milk proteins, both casein and whey, are increasingly recognized for their bioactive components which may bestow therapeutic benefits on appetite (Hartmann and Meisel 2007, Nongonierma and FitzGerald 2015, Torres-Fuentes, Schellekens et al. 2015, Nilaweera, Cabrera-Rubio et al. 2017). A casein-derived bioactive fraction with specific serotonin-2C receptor (5-$HT_{2C}$) agonist activity eliciting satiating properties in a rodent model has been described (Schellekens, Nongonierma et al. 2014). In this study, we demonstrated that a novel casein hydrolysate displayed intrinsic GHSR-1a agonist activity which translated to an effect on increasing food intake *in vivo* in rats.

The dairy hydrolysate, CasHyd, dose-dependently and specifically increased intracellular $Ca^{2+}$ in HEK293A cells heterologously expressing the GHSR-1a. We have previously reported ghrelin agonistic effects of a whey-based protein derivative in the same *in vitro* system (Howick, Alam et al. 2018). The CasHyd described here, displays superior potency (0.27 mg/ml) compared to the whey derived fraction, however it is considerably less than the endogenous GHSR-1a ligand (0.25 µg/ml), ghrelin (Figure 2.1). This is likely reflective of the fact that CasHyd is a mixture of peptides, only some, or one, of which may be active on GHSR-1a. Additionally, the *in vitro* activity is specific, with negligible effects on WT, $5HTR_{2C}$ or the fully edited form of $5HTR_{2C}$. The activity of CasHyd is also shown to be both additive and synergistic to native ghrelin *in vitro* (Figure 2.5,2.6). Furthermore, the activity of CasHyd was assessed in the neuroblastoma cell line, SHSY-5Y, engineered to overexpress the GHS-R1a receptor as a native receptor (no fluorescent tag) using lentiviral vectors. A calcium mobilization response following exposure to the

endogenous ligand ghrelin as well as the dairy-derived hydrolysate was observed in both this cell line, and that of HEK293A (Figure 2.4). This reinforces the GHS-R1a activating potential of the hydrolysate. Moreover, hydrolysate mediated GHS-R1a activation was obtained in a cultured neuroblastoma cell line expressing the GHS-R1a. This provides promising evidence to further examine CasHyd activity on GHSR-1a in a physiologically relevant environment using primary cultured neuronal cells. HPLC-SEC contrasted the size differences of the parent casein protein and CasHyd, confirming the efficacy of the hydrolyzation process (Figure 2.3). The high presence of low molecular weight peptide sequences (<1kDa, Table 2) is critical to the bioactivity reported in these assays.

This is the first instance that a casein-derived peptide has been shown to have GHSR-1a agonist properties *in vitro*. Furthermore, this *in vitro* activity has been demonstrated to translate to an increase in food intake *in vivo* in a rodent model. We show that CasHyd displays evidence of enhancing food intake in healthy SD rats. Male and female rats treated orally using a solution of CasHyd at a dose of 50mg/kg showed a three-fold increase in food intake over the six hour experiment (Figure 2.8), however this is tempered by a relatively low quantity of food consumed overall. Time bins illustrate a significant elevation in both groups in the 5-6 hour timepoint, potentially indicative of a prolonged/delayed systemic effect. Interestingly, following intraperitoneal injection of CasHyd (50mg/kg dose), neither male nor female rats displayed a significant increase in food intake relative to control (Figure 2.7). The apparent success of oral delivery of the bioactive peptide relative to injection may be reflective of the distribution of the GHSR-1a *in vivo*, which is heavily expressed in the gastrointestinal tract and involved in neuronal signalling to appetite centres in the brain (Howick, Alam et al. 2018).

Despite the apparent increase in food intake after oral gavage of CasHyd, *in vitro* assays confirm that acidic pH, comparable to that experienced in the gastric conditions, is detrimental to CasHyd bioactivity (Figure 2.9). The ability of bioactive peptides to elicit a beneficial effect *in vivo* is hence likely to be highly dependent on the use of a gastro-protective delivery system (de Vos, Faas et al. 2010, Gleeson, Ryan et al. 2016). This is in line with recent literature highlighting the role of drug-delivery

research strategies for bioactive materials (Brayden and Baird 2013, Gleeson, Ryan et al. 2016). Therefore, we sought to develop a gastro-protective formulation to minimize acid-mediated degradation of the casein fraction and enhance delivery to the small intestine. A coated pellet formulation was established, utilizing extrusion-spheronisation for pelletisation, followed by coating with an ethylcellulose-based polymer using fluidized bed technology. CasHyd encapsulated in a coated oral delivery vehicle (pellets) showed a trend towards an increase in food intake in female rats ($p=0.054$), and male rats ($p=0.097$) at the 6-hour timepoint, although overall no significant differences are noted. Furthermore, the absolute amount of food consumed in the experimental period is higher after dosing with pellets (Figure 2.12) compared with CasHyd solution (Figure 2.8), which may be reflective of the bulk volume of pellets; it may be that dosing pellets which slowly disintegrate in the intestine creates a paradoxical increase in food intake, thereby confounding any comparisons to orally dosed solutions. Furthermore, the orally dosed pellets impact on the timing of the release of bioactive which may in itself lead to different effects i.e. the immediate availability of the peptide in the stomach vs the gradual release from slowly dissolving pellets.

Overall, although food intake results showed high variability, these initial proof-of-concept studies represent promising results. The increase in food intake after oral gavage of CasHyd is tempered by efforts to substantiate the claim as an appetite stimulant by incorporating it into a gastro-protected vehicle; these efforts did not find any such increase. Further discussion on the study limitations is therefore merited, specifically in relation to the suitability of the experimental setup for assessing food intake, and peptide release characteristics from the delivery system.

Firstly, although the food intake model described has been reported in previous studies involving a bioactive peptide, food intake in rodents is inherently variable, and susceptible to change by a multitude of factors. Inter-experimental variability is evident in the differing absolute amount of food consumed between studies. Healthy, normophagic rats were used in this study; this makes it difficult to observe any increases in food intake given that metabolic drive would generate a healthy appetite by default. All experiments were also carried out in the light phase, when rodents

normally would be asleep – circadian fluctuations may serve here as a confounder to assessing true appetite. Furthermore, the dosing procedure exerted a degree of restraint stress upon the animals, while there is a risk of minor local injury to the oesophagus in gavaged rats which is also likely to impact on food intake. Secondly, the bioactive hydrolysate itself is likely to be highly fragile *in vivo*, due to low gastric pH (discussed above), as well as intestinal peptidases. Variability in results may well be a consequence of systemic breakdown. Thirdly, in the case of pelletized CasHyd, the delivery system design incorporated the peptide into a gastro-protected pellet which exerted a degree of processing stress on the peptide, resulting in a loss of ~40% bioactivity. The bulk effect of solid pellets also seems to have imparted a default increase in food intake in both males and females compared to oral solution. While this formulation was useful as proof of concept, process optimization is required to minimize activity losses, reduce bulk volume and tailor the release profile further *in vivo.*

Despite the above described caveats to this study, hitherto, a lot of evidence substantiating nutraceutical and bioactive health claims comes from *in vitro* bioinformatics, with many lacking tangible *in vivo* evidence of effect (Li-Chan 2015, Nongonierma and FitzGerald 2015). Therefore, evidence is needed to further support the claim of dairy-derived bioactives for appetite modulation. Our novel casein-derived bioactive peptide, CasHyd, shows promising results translating a specific *in vitro* bioactivity with high potency, to a promising biofunctional effect on food intake *in vivo*, suggesting overall success of this proof of concept study. Given a more suitable platform for assessment of food intake, and/or an optimized oral delivery mechanism to improve stability during formulation, a considerable potential to increase food intake *in vivo* by targeting intestinal GHSR-1a exists.

The area of bioactives for appetite modulation is of growing commercial interest and has the potential to address an unmet clinical need by providing an evidence-based, dietary incorporated, early intervention for conditions of undereating. CasHyd is a GHSR-1a agonist which represents a novel nutraceutical approach to increasing appetite in susceptible populations. However, further work must be done in

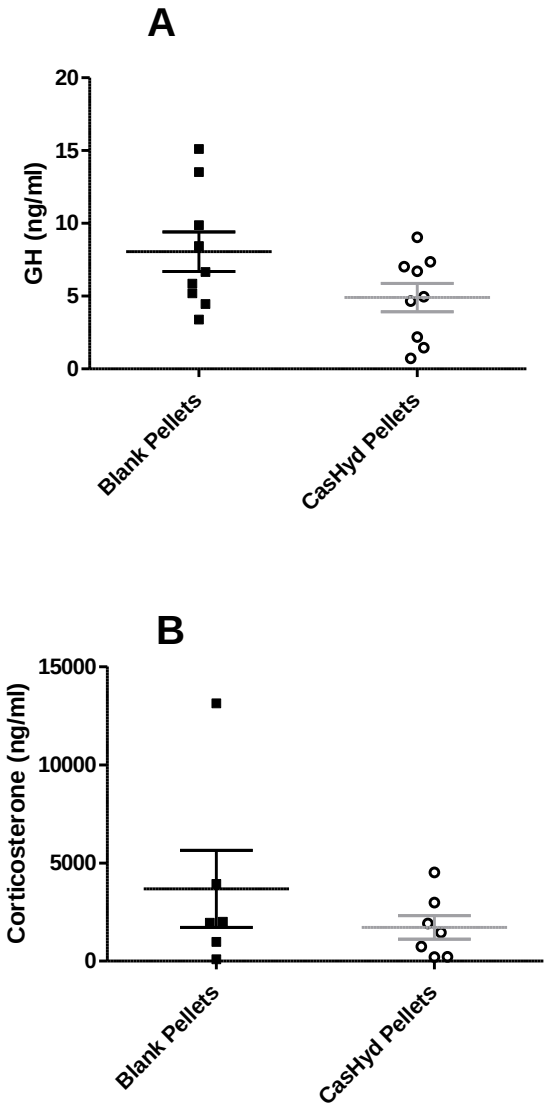
order to fully elucidate its clinical merit, while technology to retain and enhance activity *in vivo* is also required.

# Conclusion

This work describes a dairy-derived peptide with potent activity on the GHSR-1a *in vitro. In vivo* preclinical studies with this bioactive peptide show its potential to act as an appetite stimulant after oral administration. CFI was increased three-fold after 6 hours in male and female SD rats after a single oral dose. However, while activity of CasHyd was eliminated following exposure to gastric pH, administration of CasHyd in a gastro-protected pellet formulation only showed a trend towards increased food intake in both males and females. Variable results may be reflective of the suboptimal release of peptide coupled with loss of bioactivity *in vivo,* and/or potential lack of suitability of the model to assess subtle appetitive changes in a normophagic rat cohort. Overall, high *in vitro* efficacy on the GHSR-1a has translated to evidence of an effect on food intake *in vivo.* Therefore, we consider this study a valuable contribution to the growing body of evidence for nutraceuticals and nutraceutical encapsulation platforms, which serves as a useful reference for further investigations in preclinical models of age-related malnutrition or cachexia.

# Supplementary materials

## 2.25 Blood biomarker analysis



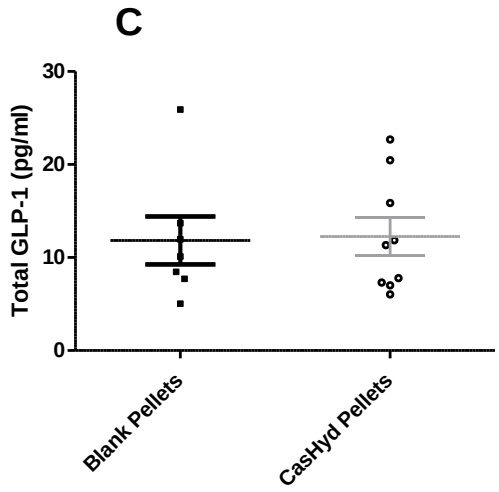



***Figure S1. Enzyme Linked Immunosorbent Assay for Growth Hormone, total GLP-1 and Corticosterone:*** *4 hours post-dosing with CasHyd pellets animals were euthanized and trunk blood collected for analysis. Growth Hormone (GH), Total Glucagon-Like Peptide (GLP-1) and Corticosterone (Cort). No significant differences were detected between the treatment and control animals 4 hours post-dosing with either CasHyd-loaded pellets or blank pellets.*

# Chapter 3

# Sustained-Release Multiparticulates for Oral Delivery of a Novel Peptidic Ghrelin Agonist: Formulation Design and *in vitro* Characterization

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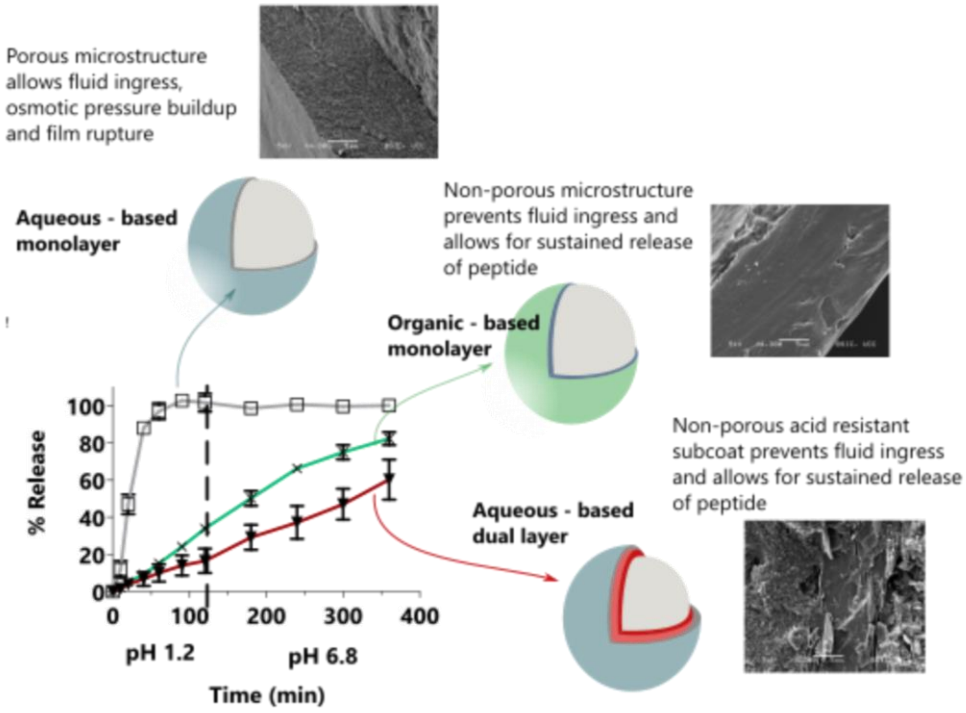
# Abstract

There is an impetus to provide appropriate sustained release oral delivery vehicles to protect biofunctional peptide loads from gastric degradation *in vivo.* This study describes the generation of a high load capacity pellet formulation for sustained release of a freely water-soluble dairy-derived hydrolysate, FHI-2571. The activity of this novel peptidic ghrelin receptor agonist is reported using *in vitro* calcium mobilization assays. Conventional extrusion spheronization was then used to prepare peptide-loaded pellets which were subsequently coated with ethylcellulose (EC) film coats using a fluid bed coating system in bottom spray (Wurster) mode. Aqueous-based EC coating dispersions produced mechanically brittle coats which fractured due to osmotic pressure build-up within pellets in simulated media. In contrast, an ethanolic-based EC coating solution provided robust, near zero-order release in both USP Type 1 and Type 4 dissolution studies. Interestingly, the functionality of aqueous-based EC film coats was restored by first layering pellets with a methacrylic acid copolymer (MA) subcoat, thereby hindering pellet core swelling in acidic media. Broadband Acoustic Resonance Dissolution Spectroscopy (BARDS) was utilized as a complementary technique to confirm the results seen in USP dissolution studies. Retention of activity of the ghrelinergic peptide hydrolysate in the final encapsulated product was confirmed as being greater than 80%. The described pellet formulation is amenable to oral dosing in small animal studies in order to assess *in vivo* efficacy of the whey-derived ghrelinergic hydrolysate. In more general terms, it is also suitable as a delivery vehicle for peptide-based bioactives to special population groups e.g. paediatric and geriatric.




***Graphical Abstract***. *Graphical synopsis of Chapter 3*

# Introduction

Oral peptide delivery remains a bottle-neck in the transition of potentially effective therapeutics from bench to bedside (Brayden and Alonso 2016). Bioavailability of peptides is consistently poor due to the acidic and enzyme-mediated degradation in gut lumen, leading to loss of efficacy. The rapid degradation of bioactive peptide structures *in vivo* necessitates drug delivery technologies which protect the payload in the gastric compartment and allow for site specific delivery to the small and large intestine (Malik, Baboota et al. 2007). Various formulation approaches have been adopted to protect peptides from degradation within the gastrointestinal tract and increase oral bioavailability, ranging from standard formulations containing functional excipients, to micro- and nano- based (colloidal) delivery systems (Lakkireddy, Urmann et al. 2016). However, commercial success in terms of an orally active peptide formulation has been limited to a few niche, high potency peptides which can achieve therapeutic efficacy with limited bioavailability (i.e. <1%) (Aguirre, Teijeiro-Osorio et al. 2016). Micro- and nano-based delivery

systems encompass a large proportion of the efforts to translate peptide functionality *in vitro* to the clinical setting. However, various limitations exist to these respective approaches: the former typically involves complex processing steps leading to peptide degradation (Witschi and Doelker 1998, Yin, Lu et al. 2008), while the latter displays poor loading capacity (1-5%), variable release characteristics and limited scalability (Redhead, Davis et al. 2001, Jain, Khar et al. 2008). Furthermore, stresses during processing, including shear forces, exposure to organic solvents and excessive drying time will adversely impact on peptide stability, as well as interactions with hydrophilic/hydrophobic interfaces. There is thus an impetus to develop more suitable oral delivery platforms to enable assessment of *in vivo* efficacy for peptidic compounds showing promise in the *in vitro* setting.

The main aim of this study is the encapsulation of a novel bioactive peptide using a traditional multiparticulate formulation approach. These coated pellets are intended for use pre-clinically to investigate bioactive functionality in rodents. In addition, from a clinical utility perspective, pelletised dosage forms offer numerous advantages such as flexible dosing and ease of administration in special population groups. Conventional formulations such as coated pellets are widely used in the pharmaceutical industry to obtain suitable release profiles for a variety of active pharmaceutical ingredients (API) (Lecomte, Siepmann et al. 2004, McGinity and Felton 2008) . Millimetre size-range pellets have notable advantages compared to sub-micron and colloidal delivery approaches. A narrower particle size distribution allows for homogeneous film formation and more reproducible release profiles. Higher peptide loading can typically be achieved by inclusion of a pelletisation aid such as microcrystalline cellulose. The process is readily scalable to industrial size, while critically this represents a flexible dosing platform ranging from pre-clinical proof of concept studies, to clinical dosing in special population groups, i.e. paediatric and geriatric patients. Fluid bed coating technology holds many advantages for coating peptide-loaded matrix pellets. This is a well-established process that allows for simple and efficient polymer layer deposition and subsequent reliable delayed/sustained drug release, depending on the nature of the functional polymeric coat applied. Furthermore, the processing conditions are mild relative to other methods such as pan coating, while low weight gains achieve reliable, uniform coating. Typically, pellets

are fluidized by high flow air, while an atomized coating solution or suspension is pulsed onto the pellets. As liquid coating material is deposited and simultaneously dried, the latent heat of evaporation of solvent means that the microenvironment of each individual pellet is considerably lower than the pre-heated inlet air (El Mafadi, Picot et al. 2005, Poncelet D 2009).

The therapeutic potential of bioactive peptides for treating many health problems, including appetite-related disorders, is becoming increasingly apparent (Torres-Fuentes, Schellekens et al. 2015). Recent work in rodents has demonstrated the ability of whey protein isolate to reduce the expression of satiating genes in the hypothalamus, thereby increasing energy intake (Nilaweera, Cabrera-Rubio et al. 2017). This study describes a novel peptidic dairy hydrolysate, FHI-2571, with ghrelin receptor agonist activity. Ghrelin, a 28-amino acid containing peptide, is produced in the stomach and functions as an endogenous appetite-stimulant (Kojima, Hosoda et al. 1999, Nakazato, Murakami et al. 2001, Howick, Griffin et al. 2017). The ghrelin receptor has thus been a pharmacological target to reduce appetite in obesity as well as to stimulate food intake in conditions of malnutrition and cachexia (wasting syndrome) (Schellekens, Dinan et al. 2010, Howick, Griffin et al. 2017). While the precise site of action of ghrelin is still open to some debate (Howick, Griffin et al. 2017), the high prevalence of the ghrelin receptor throughout the small and large intestinal mucosa is thought to facilitate communication with appetite centres in the brain via the vagus nerve (Date 2012), and thus may hold potential as a local therapeutic target (Lakkireddy, Urmann et al. 2016).

Overall, this study aims to first assess the *in vitro* efficacy of a novel ghrelin receptor agonist, FHI-2571, and investigate a formulation approach to progress this bioactive to *in vivo* studies. To overcome the acidic and proteolytic degradation of this whey-derived hydrolysate in the stomach and upper intestine, we have developed a sustained-release oral delivery system to minimize exposure to gastric acid and intestinal peptidases. *In vitro* release profiles of FHI-2571 in traditional USP dissolution tests, confirmed using BARDS, demonstrate the capability of our formulation approach in achieving prolonged, elevated levels of bioactive throughout

the small intestine *in vivo*. Activity assays confirm that the peptide retains good bioactive functionality post-encapsulation.

# Materials and Methods

## 3.1 Materials

Dairy-derived peptide hydrolysate (FHI-2571) was provided by Food for Health Ireland (see section 2.2). Methacrylic acid and ethyl acrylate copolymer type C (MA, Acryl-EZE® II) and ethylcellulose (EC) (Ethocel™ Standard 20 Premium) were both purchased from Colorcon Corp., Dartford, Kent, UK, while aqueous pseudo-latex of EC (Surelease® Type B NF) was sourced from Colorcon Corp., Indianapolis, IN, USA. Microcrystalline cellulose (MCC, Avicel® PH-101 NF Ph. Eur.) was purchased from FMC Corp., Little Island, Cork, Ireland. Pharmaceutical grade ethanol 96% (v/v) was procured from Carbon Chemicals Group Ltd., Ringaskiddy, Cork, Ireland. Unless otherwise stated, only deionised water was used in this study. For the $Ca^{2+}$ mobilization assays, fetal bovine serum (3.3%) was obtained from Sigma-Aldrich, Arklow, Wicklow, F7524. Assay buffer was composed of 1x Hanks balanced salt solution, HBSS, Gibco™ 14065049 (Thermo Fisher Scientific™), containing 20 mM HEPES (Sigma-Aldrich, Arklow, Wicklow, H0887). The endogenous agonist, ghrelin (rat), was supplied by Tocris Bioscience, Avonmouth, Bristol, UK (Cat. No. 1465).

## 3.2 Generation of FHI-2571

A dairy-peptide hydrolysate was prepared by a method similar to a previously published method (Mukhopadhya, Noronha et al. 2015). Briefly, bovine milk derived whey protein (80 % w/w protein, Carberry Group, Ballineen, Cork, Ireland) was suspended at 10 % protein (w/w) in reverse osmosis-treated water and agitated continuously at 50 °C for 1 h in a jacketed tank. The pH was adjusted using a NaOH 4.0 N solution (VWR, Dublin, Ireland). A bacterial food-grade enzyme preparation, was added to the protein solution until 7-12 % degree of hydrolysis was achieved. The enzyme was then inactivated by heat-treatment and the resultant hydrolysate solution was dried in a Niro TFD 20 Tall-Form Dryer (GEA, Düsseldorf, Germany).

## 3.3 $Ca^{2+}$ mobilization assay for peptide ghrelin receptor activity pre- and post-encapsulation

Ghrelin receptor mediated changes in intracellular $Ca^{2+}$ mobilization were monitored on a FLIPR Tetra High-Throughput Cellular Screening System (Molecular Devices Corporation, Sunnyvale, California, USA). $Ca^{2+}$ mobilization assays were performed according to the manufacturer's instructions and modified from a previously described method (Pastor-Cavada, Pardo et al. 2016). Human Embryonic Kidney (HEK293A) cells stably transfected with the ghrelin receptor were seeded in black 96-well microtiter plates at a density of 3 x $10^4$ cells/well and maintained overnight at 37 °C in a humidified atmosphere containing 5 % $CO_2$. Twenty-four hours before the experiment, media was replaced with serum-free media containing 1 % non-essential amino acids (NEAA). On experimental day cells were incubated with 80 µL of 1xCa5 dye in assay buffer (1x Hanks balanced salt solution - HBSS, supplemented with 20 mM HEPES buffer) according to the manufacturer's protocol (R8141, Molecular Devices Corporation, Sunnyvale, CA). Addition of compound (40 µL/well) was performed by the FLIPR Tetra, and fluorescent readings were taken for 120 seconds at excitation wavelength of 485 nm and emission wavelength of 525 nm. The relative increase in cytosolic $Ca^{2+}$ was calculated as the difference between maximum and baseline fluorescence and depicted as percentage relative fluorescent units (RFU) normalized to maximum response (100 % signal) obtained with 3.3 % fetal bovine serum (FBS). Background fluorescence was recorded in cells in assay buffer alone and subtracted from RFUs. All compounds and hydrolysates used in experiments were prepared in assay buffer. FBS (3.3 %) and the endogenous agonist ghrelin (1465; Tocris) were used as positive controls of $Ca^{2+}$ influx. Responses were considered as positive when $Ca^{2+}$ influx exceeds 20% compared to control. Data were analysed using GraphPad Prism software (PRISM 5.0; GraphPAD Software Inc., San Diego, California, USA). Sigmoidal concentration-response curves were generated using nonlinear regression analysis with variable slope.

## 3.4 Pellet preparation by extrusion-spheronisation

Initial process optimization studies, focusing on pellet production, trialled a range of different ratios of FHI-2571 to MCC (50:50, initially). The gradual reduction

of FHI-2571 content from 50% afforded a robust pellet with minimal generation of fines at 33% peptide loading (data not shown). Therefore, requisite quantities of FHI-2571 and MCC were combined in a ratio of 33:67, respectively, and manually blended for 1 minute. The powder was then added to a Kenwood Major planetary mixer (KM005, Kenwood Ltd., Hampshire, UK), fitted with a 'K' blade mixing arm, and dry blended at minimum setting for 5 minutes. The resultant dry powder blend was granulated by addition of deionised $H_2O$, under constant agitation by planetary mixer at minimum setting. A homogenous dispersion of moisture was ensured, by adding deionised$H_2O$ periodically from an atomizer every 15 seconds. Mixing was stopped every 2-3 minutes, to scrape material down from the sides of the mixing vessel. The granulation end-point was achieved upon addition of a cumulative amount of deionised$H_2O$ equivalent to 45 % (w/w) of the dry powder blend. The granulate was immediately extruded at an extrusion speed of 17 – 19 rpm using a sieve extruder (Caleva® Extruder 20, Caleva Process Solutions, Sturminster Newton, Dorset, UK). Both the screen thickness and aperture diameter were 1 mm. The extrudate was subsequently spheronized for 1.5 minutes at a speed of 1500 rpm, using a Caleva® Spheroniser 250 (Caleva Process Solutions, Sturminster Newton, Dorset, UK) equipped with a cross-hatch friction plate having a diameter of 22.5 cm. Resulting pellets (c. 100 g) were dried in a laboratory scale microfluid bed system (Vector Corp., Marion, IA, USA) at 40 °C for 20 minutes and then stored at room temperature in an airtight container until further testing took place.

## 3.5 Pellet film coating

Film coating was performed in a laboratory scale microfluid bed system, equipped with a Wurster funnel insert, in bottom-spray mode. Both nozzle air (16.6 – 16.7 psig) and airflow (310 – 335 L/minute) were adjusted to maximum setting. Liquid feed rate (gram/minute) and spray pattern parameters varied, depending on the film coating polymer mixture (see Table 1). Various coating polymer mixtures were prepared; a concentrated 25 % (w/w) aqueous pseudo-latex of EC (Surelease® Type B) was diluted to 11 % (w/w), using deionised $H_2O$ water. Dissolution of EC (Ethocel™ Standard 20 Premium) in 96 % (v/v) EtOH to produce a 5 % (w/w) ethanolic EC polymer coating solution was performed slowly under conditions of

vigorous agitation. Dry methacrylate powder (Acryl-EZE® II) was gradually dispersed in deionised $H_2O$ water to produce a 10 % (w/w) coating mixture. All of the coating polymer mixtures were subjected to constant agitation at 900 rpm, for not less than 30 minutes, using a magnetic stirrer at room temperature. Agitation of the polymer coating mixtures (750 – 850 rpm at room temperature) was maintained during film coating procedures. Uncoated pellets were charged to the coating vessel (25 – 50 g, pellet load), and the coater reassembled. Pellets were pre-heated in-situ, for approximately 10 minutes (inlet air temperature 80 °C; outlet air temperature ~ 50 °C), prior to commencing film coating. The amount of coating polymer required for film coating was based on a pre-determined weight gain, based on dry uncoated pellet mass.

***Table 3.1. Coating parameters.*** *Coating parameters employed during the film coating with methacrylic acid and ethyl acrylate copolymer type C (Acryl-EZE® II), aqueous dispersion of ethylcellulose (Surelease® Type B), and organic solution of ethylcellulose (Ethocel™ Standard 20 Premium) polymer mixtures, respectively.*

| **Film Coating Parameter** | | **10 % (w/w) Acry-LEZE® II in water** | **11 % (w/w) Surelease® Type B in water** | **5 % (w/w) Ethocel™ in 96 % (v/v) EtOH** |
|---|---|---|---|---|
| Liquid Feed Rate (g/minute) | | 0.8 | 1.0 | Minimum |
| Spray Pattern | On (minute) | 0.4 | 0.4 | Continuous |
| | Off (minute) | 0.1 | 0.1 | |

The duration of the film coating process was determined by the theoretical percentage of coating required, and the dry weight of pellets added to the spray coater.

The vessel containing the coating polymer mixture was weighed before and after the coating process, to determine the actual weight of coating solution sprayed onto the pellets. The microfluid bed coating system was visually monitored to ensure that a steady uniform flow of pellets was maintained within the spray chamber.

## 3.6 pH susceptibility tests

Powdered FHI-2571 (6 g) was dissolved in 100 ml of deionised $H_2O$ and aliquoted into 4 x 25 ml samples. Next, 3 M HCl was added to bring the individual solutions to the requisite pH (pH 1, 3, 5 and untreated), using a pHenomenal® 1000L pH meter with a pHenomenal® 221 pH electrode. After pH was adjusted, samples were incubated for 30 minutes under gentle agitation. Finally, 50 µL of each sample was removed and added to 950 µL of $Ca^{2+}$ assay buffer and neutralization of acidic pH confirmed before samples were added to cells.

## 3.7 *In vitro* dissolution tests

### 3.7.1 USP Type 1 (Basket) Dissolution studies

Dissolution testing (USP Type 1) was performed, using a basket-type dissolution apparatus (DISTEK, Inc., Model 2100C, North Brunswick, NJ, USA) with 500 mL of both simulated gastric fluid sine pepsin (SGFsp) (pH 1.2) and simulated intestinal fluid sine pancreatin (SIFsp) (pH 6.8) as dissolution media. Dissolution bath temperature was maintained at 37 ± 0.5 °C. Impeller shaft speed was 50 rpm. Dissolution medium sampling was conducted at predefined timepoints (10, 20, 40, 60, 90, 120, 180, 240, 300, and 360 minutes) from a location not less than 1 cm from the vessel wall and midway between the top of the rotating impeller and dissolution media surface. After sampling, an equal volume of dissolution medium was added to the dissolution vessel.

### 3.7.2 USP Type 4 (Flow-through) Dissolution studies

An Erweka® flow-through apparatus (Model DFZ 720, ERWEKA GmbH, Germany), equipped with a HKP 720 piston pump and 22.6 mm diameter cells, was used to perform USP type 4 dissolution studies. The temperature of the water bath was maintained at 37 °C. Experiments were carried out over six hours using the closed loop system at a flow rate of 4 ml/minute. The dissolution media was composed of 100 mL SGFsp for the first two hours. SGFsp was then replaced with 100 mL of SIFsp, after two hours. Samples (1 mL) were taken at the same time intervals as for USP Type 1 dissolution (described above). After sampling, an equal volume of dissolution medium was added to the dissolution vessel

## 3.8 Peptide quantification assay

The bicinchoninic acid (BCA) assay was performed using a BCA assay kit (Thermo Fisher Scientific™ Pierce™ BCA Protein Assay, Catalog Number 23225) according to a previously published method. Diluted stock samples were made using a 2 mg/ml stock solution of FHI-2571 in SGFsp. Using this stock solution, a serial dilution was performed to afford six 0.1 mL solutions with concentrations of 2, 1, 0.5, 0.25, 0.125, and 0.0625 mg/mL, respectively. This method was repeated, using SIFsp. A 25 µL volume of each sample obtained during dissolution testing (see section 2.6.) was then transferred to a 96-well plate. After the dissolution experiment was completed, peptide not released from the formulation was determined. Remaining pellets were removed, physically crushed and redissolved in the relevant media. The quantity of liberated peptide was determined using the BCA assay. Working reagent was prepared by mixing BCA assay Reagent A with BCA assay Reagent B in a ratio of 50:1. The working reagent (200 µL) was then transferred to each well. The plates were then covered and incubated at 37 °C for 30 minutes. Spectrophotometric analysis (Flexstation II Multiplate Fluorometer, Molecular Devices, Sunnyvale, California) was performed at 562 nm. A standard curve was made by plotting the average blank-corrected absorbance (562 nm) for each BCA assay standard versus concentration (mg/mL). The standard curve was used to determine the protein concentration of each unknown sample.

## 3.9 Electron microscopy

Samples were mounted onto aluminium stubs using double sided carbon tape. All samples were sputter coated with a 5 nm layer of gold palladium (80:20) using a Quorum Q150 RES Sputter Coating System (Quorum Technologies, UK), before being examined using a JEOL JSM 5510 Scanning Electron Microscope (JEOL Ltd., Japan) in the BioSciences Imaging Centre, Department of Anatomy & Neuroscience, UCC. Digital electron micrographs were obtained of areas of interest.

## 3.10 Broadband Acoustic Resonance Dissolution Spectroscopy

A Broadband Acoustics Resonance Dissolution Spectroscopy (BARDS) , as described previously by Fitzpatrick *et al.* (Fitzpatrick, Evans-Hurson et al. 2014) was used to investigate the BARDS responses (BARDS ACOUSTIC SCIENCE LABS, Cork, Ireland). A sample size of 0.1 g was used in each experiment. Testing was performed under acidic conditions, using 25 mL simulated gastric fluid (SGFsp pH 1.2) as solvent. The stirrer rate was set to 300 rpm. Prior to testing, temperature (c. 25.5 °C), relative humidity (c. 47%), and pressure (c. 1025 mbar) were recorded. Before sample addition, steady state resonances were recorded for 30 s, while the magnetic follower was in motion. Spectra were recorded using a microphone (Sony ECM-CS10, range 100 Hz – 16 kHz) for 560 – 1300 s. The frequency time course of the fundamental frequency curve is shown as manually extracted data from the recorded acoustic response. All experiments were performed in triplicate ($n$ = 3) and average values with error bars representing the standard deviation are presented.

## 3.11 Data Analysis

Data were analysed and graphs generated using both GraphPad Prism software and Microsoft Excel software. All means were calculated from the results of at least three independent experiments carried out in triplicate. For the *in vitro* calcium mobilization assays, standard error of the mean (SEM) is depicted, while all dissolution results report standard deviation (SD).

# Results

## 3.12 A dairy-derived peptide exhibits ghrelin receptor agonist activity *in vitro*.

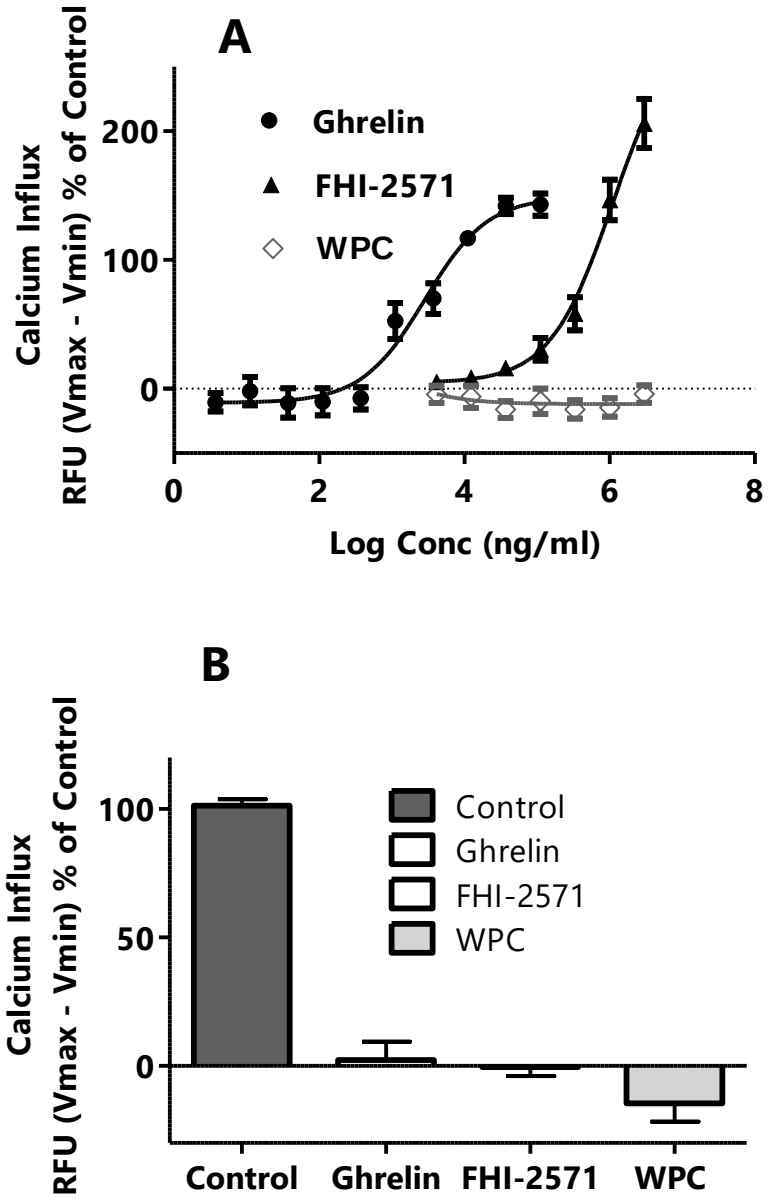



***Figure 3.1A. Concentration response curve of novel whey-derived hydrolysate****. Concentration response curves for the endogenous ghrelin receptor ligand, ghrelin, the whey-derived hydrolysate, FHI-2571 and the parent whey protein concentrate (WPC) measured in ghrelin receptor over-expressing HEK293A cells. Intracellular $Ca^{2+}$ increase was depicted as a percentage of maximal $Ca^{2+}$influx in relative fluorescence unit (RFU) as elicited by control (3.3% FBS). Graph represents mean ± SEM of three independent experiments performed in triplicate.* ***Figure 3.1B****:* ***No***

***activity of test compounds on wild-type cells.*** *Activity of control (FBS), ghrelin (1uM), FHI-2571 (3mg/ml) and parent whey protein concentrate (WPC) (3mg/ml) in wild-type (HEK293A-WT) cells (representative of three independent experiments carried out in triplicate).*

The activity of the whey-derived hydrolysate, FHI-2571, on the ghrelin receptor was shown using intracellular $Ca^{2+}$ mobilization, as a measure of downstream ghrelin receptor signalling activation (Schellekens, van Oeffelen et al. 2013), in HEK293A cells (human embryonic kidney cells) stably expressing the ghrelin receptor tagged with an enhanced green fluorescent protein (GHSR-1a-EGFP) (Figure 3.1A). FHI-2571 hydrolysate stimulated calcium mobilization in cells expressing ghrelin receptor in a concentration-dependent manner, with the $EC_{50}$ = 1.1mg/ml and efficacy ($E_{max}$) reaching 205%. The potency of FHI-2571 is 1000-fold lower compared to the endogenous receptor ligand, ghrelin ($EC_{50}$ = 2.84µg/ml). Interestingly, the maximal response attained for FHI-2571 hydrolysate is higher compared to ghrelin ($E_{max}$ = 150%). Efficacy of both compounds was normalized to the maximal response of the positive control (3.3% FBS, $E_{max}$ = 100%). Critically, the concentration response curve shows that the FHI-2571 hydrolysate has ghrelin receptor agonist activity, while the un-hydrolysed parent whey protein concentrate (WPC) fails to elicit appreciable activity in the same assay (Figure 3.1A). Furthermore, no $Ca^{2+}$ influx was observed in wild-type HEK293A cells (HEK293A-WT) not expressing the ghrelin receptor, when exposed to the FHI-2571 hydrolysate, while treatment with control (FBS) resulted in a non-specific maximal intracellular $Ca^{2+}$ mobilization in this cell line (Figure 3.1B), indicating the specificity of FHI-2571 activity on the ghrelin receptor.

## 3.13 pH susceptibility of FHI-2571

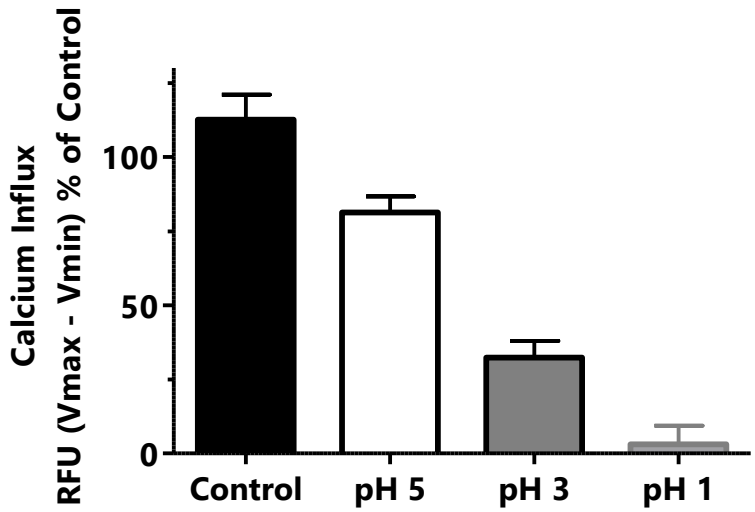



***Figure 3.2. Ghrelin receptor agonist, FHI-2571 displays pH dependent activity****. Graph represents three independent experiments carried out in at least triplicate. Reduction in FHI-2571-mediated ghrelin receptor activation following exposure to acidic pH confirms the need for an oral delivery mechanism (Control = FHI-2571 not exposed to acidic pH (3mg/ml), RFU = Relative Fluorescence Units)*

The FHI-2571 hydrolysate was exposed to varying degrees of acidic pH for a time representative of minimum gastric residence time in the fasted state (minimum 30 minutes (Tuleu, Andrieux et al. 1999)). A pH dependent loss in peptide activity is observed for the whey-derived FHI-2571 (Figure 3.2), confirming the requirement for a protective film coat to minimize exposure to gastric acid before progression to *in vivo* efficacy studies.

## 3.14 Aqueous-based Ethylcellulose dispersion yields a mechanically weak film coating

USP Type 1 (Basket) dissolution studies were carried out in both simulated gastric conditions (simulated gastric fluid, SGFsp, pH 1.2) in order to assess the release profile of peptide from the pellets. Pellets displayed burst release of the peptide, with > 80 % release over the first 60 minutes. This occurred independent of coating thickness, as 10 % coating represented no additional benefit to the 5 % (Figure 3.3). Visual investigation showed film disintegration or "shelling" occurred within 20 minutes of exposure to the aqueous medium. This was attributed to the relatively low

mechanical strength of the film coat produced from aqueous dispersion-based fluidized coating (Lecomte, Siepmann et al. 2004, Siepmann, Siepmann et al. 2008). An aqueous-based dispersion is hence an unsuitable coating approach to achieve sustained delivery of whey-derived FHI-2571 hydrolysate.

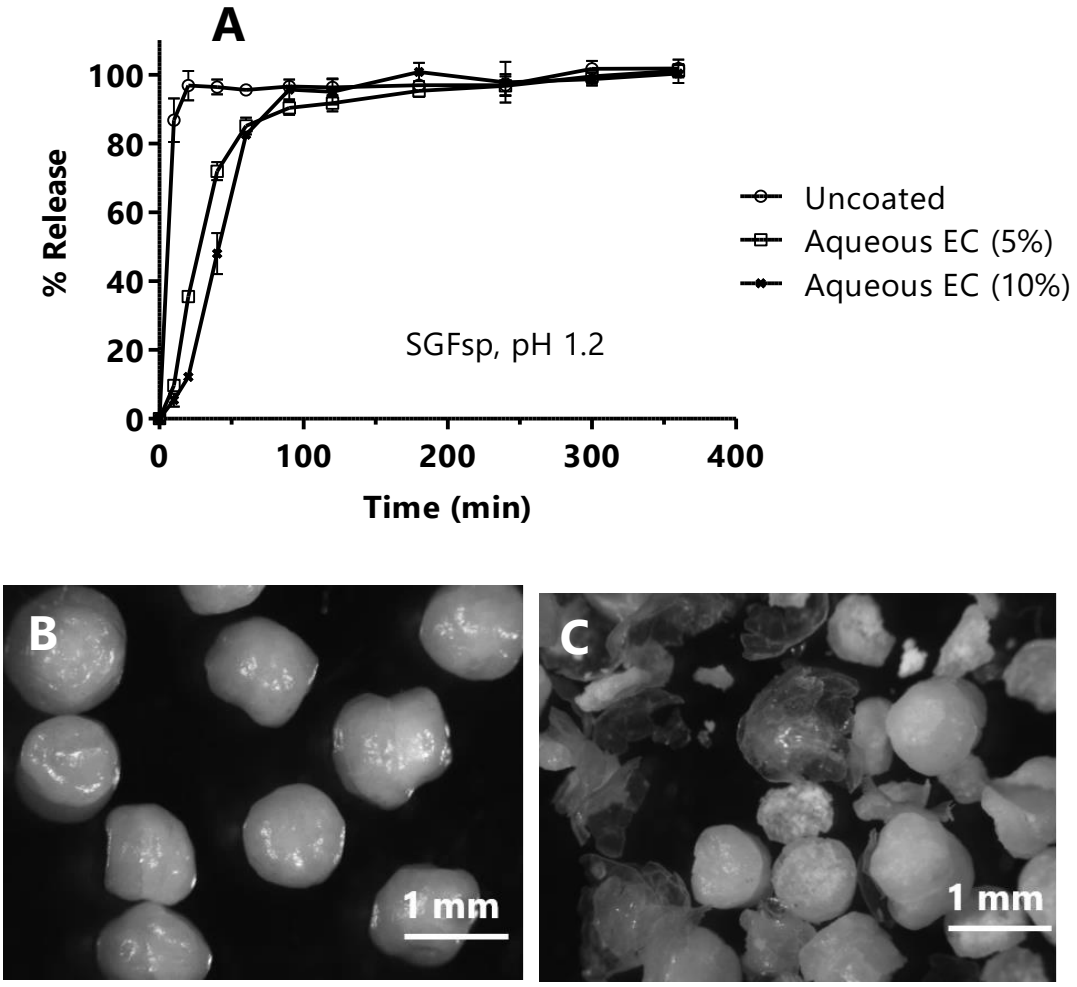



***Figure 3.3. Dissolution study of aqueous-based Ethylcellulose and FHI-2571 hydrolysate pellets.*** *USP Type 1 (Basket) dissolution studies (50 rpm, 37.5 °C) showed a burst release in simulated conditions with >80% release over the first hour in both uncoated and coated FHI-2571 hydrolysate pellets following exposure to Simulated Gastric Fluid sine pepsin (SGFsp) pH 1.2 (A). Graph represents three independent experiments carried out in triplicate. Macroscopic investigation showed an unexpected disintegration or "shelling" of the coat from the pellets, resulting in rapid release of peptide (before introduction of media, B, and after, C).*

## 3.15 Towards pellet coating achieving sustained release of peptide - Ethanolic solutions of ethylcellulose vs. aqueous dispersion based dual-coat

Two alternative strategies were employed to circumvent the observed film disintegration for EC coats prepared using aqueous-based EC dispersions (Figure 3.4). Firstly, an ethanolic solution of EC was prepared and applied to the pellets. The EC coated pellets prepared from ethanolic solutions achieved near-zero order delayed release in simulated USP Type 1 (Basket) dissolution studies carried out in SGFsp. Due to the drawbacks associated with organic solvent use (Muschert, Siepmann et al. 2009, Srivastava and Mishra 2010), an aqueous-based dual coat approach was trialled as an alternative. A pH-resistant methacrylic acid copolymer subcoat was applied to the pellets in order to prevent water ingress to the pellet core under acid conditions. Interestingly, this aqueous-based dual coat approach achieved a similar delayed release profile as the organic EC coat.

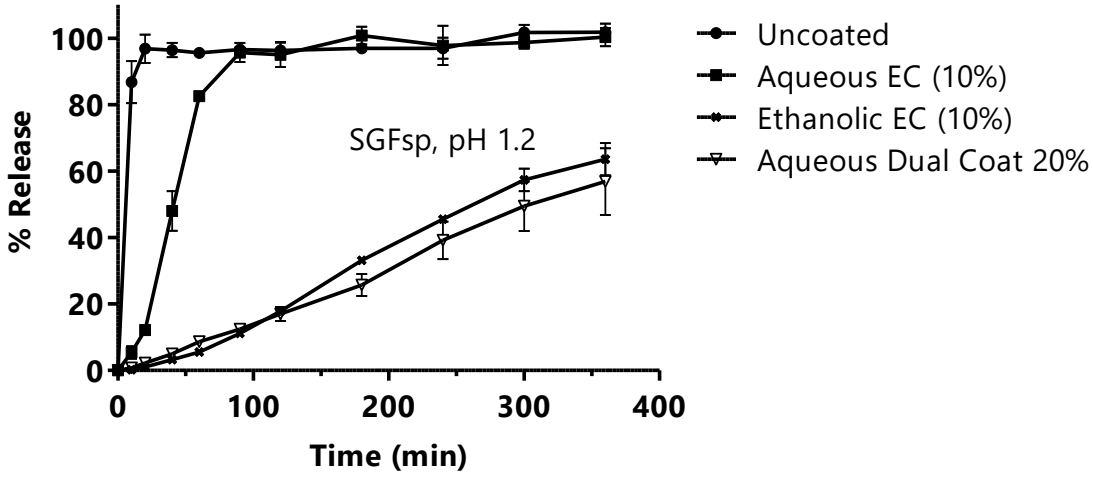



***Figure 3.4. Dissolution study of FHI-2571 hydrolysate pellets with ethanolic-based ethylcellulose film coat and aqueous-based dual coat.*** *Two different approaches were taken to circumvent the phenomenon of "shelling" as a result of coating with an aqueous dispersion of EC. 1) An organic solution of EC was applied to create a more robust coat, and 2) a methacrylic acid copolymer was layered beneath the aqueous EC to form a functional acid-resistant subcoat. USP Type 1 (Basket) dissolution studies (simulated gastric fluid, SGFsp, pH 1.2) showed effective delayed release for both organic EC and aqueous dual-coated pellets. Graph represents three independent experiments carried out in triplicate.*

## 3.16 Scanning electron microscopy investigation of whole and cross-sectioned pellets

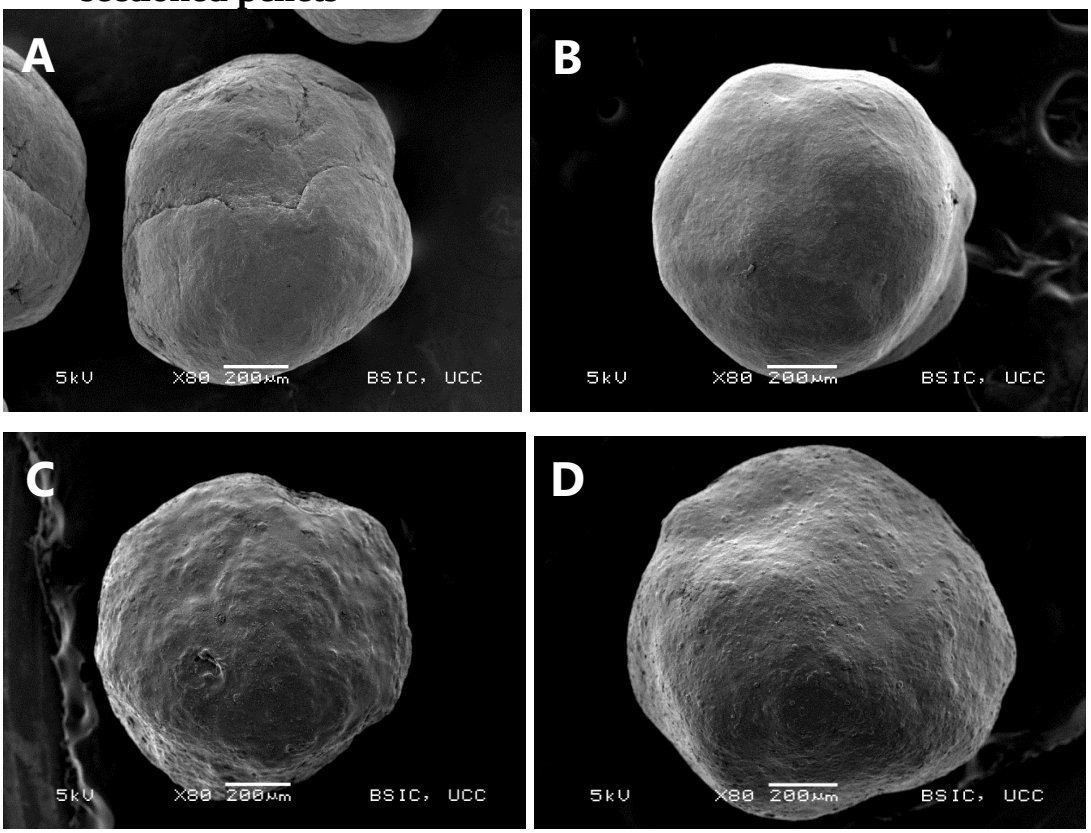



***Figure 3.5. Scanning Electron Microscope (SEM) images of whole pellets.*** *SEM images of uncoated (A), aqueous ethylcellulose (EC) coated (B), ethanolic EC coated (C) and dual-coated pellets (D).*

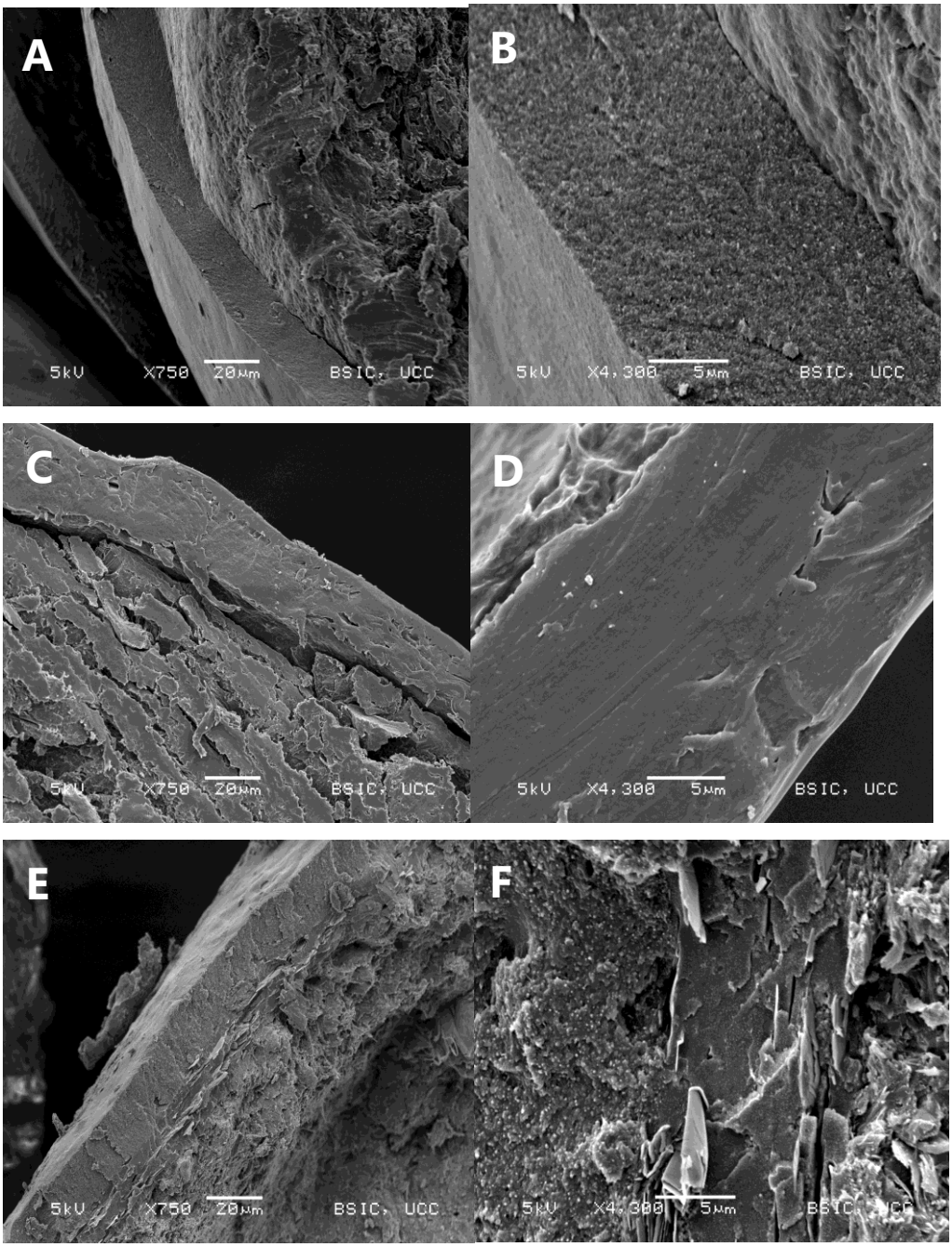



***Figure 3.6. Scanning Electron microscope (SEM) images of cross-sectioned pellets****. Aqueous EC coated pellets have a distinctly porous coat which allows for water ingress and increased osmotic pressure within the pellet, leading to swelling and rupture of the film coat (A,B). Organic EC coated pellets display a more complete, non-porous coat (C,D), while the aqueous dual coated pellets show a distinct double layer, with the porous EC overcoat and an impervious, acid resistant subcoat (E,F).*

Figure 3.5 represents SEM images of uncoated, aqueous EC coated, ethanolic EC coated and dual-coated pellets (A, B, C & D, respectively). Notably, images reveal no obvious structural cracks or pores on the surface of the coated pellets which may explain the fluid ingress and film coat rupture in the aqueous-based EC coated pellets. Figure 3.6 presents the SEM images obtained from cross-sectioned pellets with the various film coats. The aqueous EC coated pellet displays a porous cross-sectioned coat (Figure 3.6B). The porous nature of this coat can also be seen in the outer layer of the dual coated pellets (Figure 3.6F). By contrast, the ethanolic-based EC coat is distinctly non-porous and waxy in appearance (Figure 3.6D) while the methacrylate-based subcoat is also visibly non-porous (Figure 3.6F). The porous nature of the aqueous-based EC coat is likely responsible for the osmotic-induced fluid ingress to the pellet core, and subsequent film disintegration. Both the ethanolic based EC coat and the methacrylate based subcoat are functionally resistant to water ingress in simulated gastric conditions. This is attributable to the non-porous substructure evident in the photomicrographs.

## 3.17 Investigation of peptide release in pH transfer model

A pH transfer model was utilized in order to investigate whether there was any appreciable difference in release when intestinal pH was introduced to the pellets after 2 hours exposure to SGFsp (pH 1.2) conditions (Figure 3.7). Predictably the aqueous EC-coated pellets display a burst release of peptide with > 80 % release in the first hour. Notably, the dual-coated EC pellets retain a sustained release profile after the transition from acid pH, to intestinal pH (pH 6.8). In contrast to the release profiles observed under USP Type 1 conditions, the ethanolic-based EC coat also displays a faster release rate compared to the dual-coat. This difference in release patterns between Type 1 and Type 4 may be attributed to different flow patterns and agitation between the systems.

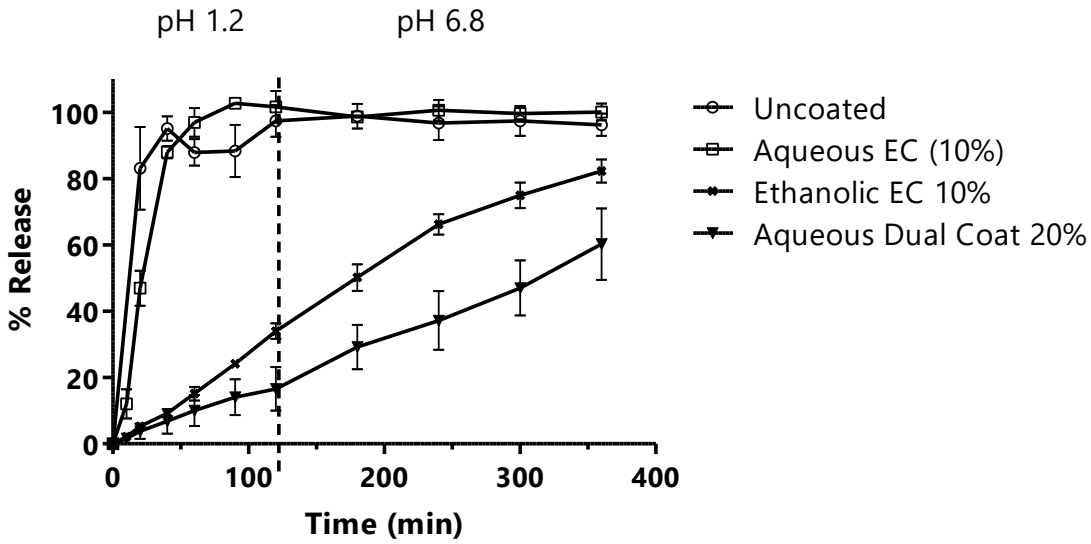



***Figure 3.7. Modelling of release profiles in a pH transition setup.*** *USP Type IV (Flow-through) dissolution experiments were carried out using 22.6 mm diameter cells to quantify FHI-2571 release in a pH-transfer model (pH 1.2 to pH 6.8). A closed loop system maintained at 37 °C recirculated 100 ml of media at 4 ml/minute for the duration of the experiment (adapted from a previous study (Keohane, Rosa et al. 2016). This yielded a predictable sustained release for both organic EC coated and dual-coated pellets. Graph represents three independent experiments carried out in triplicate. Dashed line indicates transition from SGFsp (pH 1.2) to SIFsp (pH 6.8) at 120 minutes.*

## 3.18 Broadband Acoustics Resonance Dissolution Spectroscopy (BARDS)

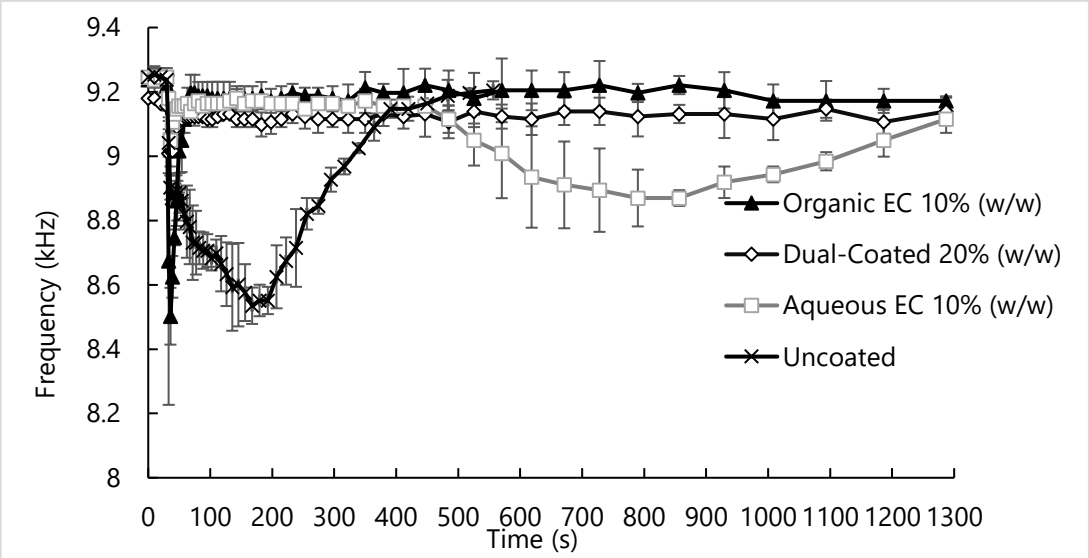



***Figure 3.8. Fundamental curve frequency of coated pellets in SGF.*** *Fundamental curve frequency time course of uncoated (Δt = 167 s), aqueous-based EC coated (10% w/w) (Δt = 790 s), dual-coated (MA and aqueous EC, 10% w/w, respectively), and organic-based EC (10% w/w) pellets, containing FHI-2571, in 25 mL SGFsp (pH 1.2). This data is representative of three independent experiments carried out, and demonstrates the comparable integrity of the dual-coated pellets with that of the organic-based EC pellets, in low pH conditions. Δt denotes the time at which minimal frequency reached (release) is reached.*

As expected in the case of uncoated pellets, a distinct change in real-time compressibility of the solvent is evident immediately after addition of the pellet to the solvent with the fundamental curve approaching its respective frequency minimum within 170 seconds (Figure 3 8). This reflects the rapid disintegration/dissolution of the pellets lacking a protective film coat. In the case of both the dual-coated pellets and organic-based EC-coated pellets, there is no noticeable dissolution/disintegration event, following addition to the acidic solvent in the vessel (SGFsp pH 1.2). This spectral observation was confirmed by visual inspection; both the dual-coated pellets and organic-based EC-coated pellets remained intact during testing and no disintegration was observed. In the case of the aqueous-based EC coated pellets, a distinct change in solvent compressibility was observed from 400 seconds, indicating that the coated pellets undergo a dissolution/disintegration event here under acidic conditions. This was also evident visually in the solvent vessel with pellets undergoing disintegration. The aqueous-based EC coated pellets demonstrated a lag time, prior to

disintegration, where the frequency minimum is observed at 780 seconds. This result indicates that the aqueous-based EC pellets do not remain intact in an acidic environment and further support findings from previous dissolution experiments (see Figure 3.3A, 3.4, and 3.7).

## 3.19 Ghrelin receptor activity post-encapsulation

In order to quantify the impact of processing conditions on bioactivity of the peptide cargos, activity of the encapsulated peptide *in vitro* was reassessed. Activity of the FHI-2571 hydrolysate liberated from the encapsulated product was determined relative to activity of non-encapsulated FHI-2571 peptide in the ghrelin receptor overexpressing cells, as before. Activity was quantified as being greater than 80% for organic EC coated and dual coated pellets (Figure 3.9).

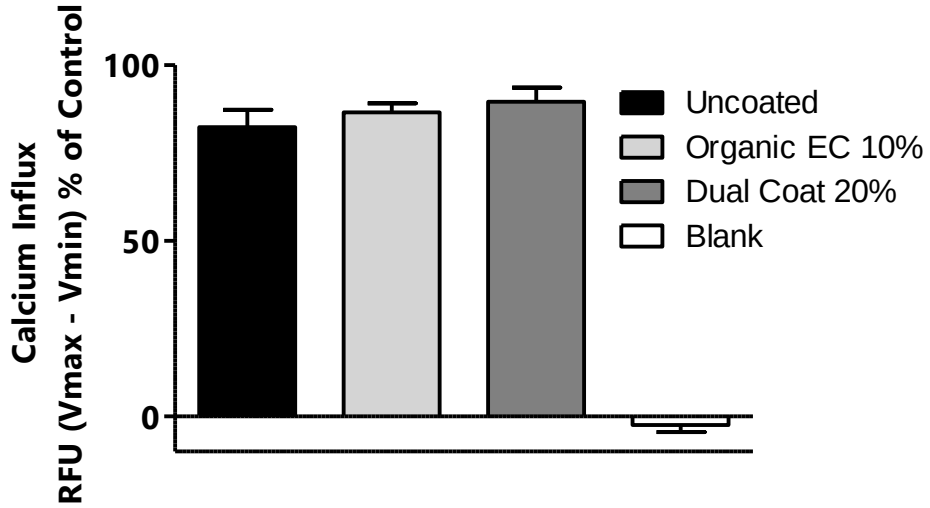



***Figure 3.9. FHI-2571 retains bioactive functionality after encapsulation.*** *Activity of FHI-2571 after encapsulation was determined relative to activity of non-encapsulated FHI-2571 on ghrelin receptor overexpressing HEK293A cell line (representative of four independent experiments carried out in at least triplicate). Activity was quantified as being greater than 80% for organic EC and dual coated pellets.*

# Discussion

Dairy-derived peptides are increasingly recognized for their bioactive components which may bestow clinical benefits (Hartmann and Meisel 2007, Torres-Fuentes, Schellekens et al. 2015). Peptides fractions have been isolated with ACE-inhibitory action, and blood-pressure lowering properties of these dairy-derived bioactives *in vivo* have been reported. Furthermore, a casein-derived bioactive fraction with specific serotonin-2C receptor (5-$HT_{2C}$) agonist activity eliciting satiating properties in a rodent model has been described (Schellekens, Nongonierma et al. 2014). Ghrelin and the growth hormone secretagogue receptor (GHSR-1a) or ghrelin receptor, play an important role in energy balance and appetite regulation (Schellekens, Dinan et al. 2010, Howick, Griffin et al. 2017). Many studies have reported potent appetite-stimulating effects of both peripheral and central administration of ghrelin (Tschop, Smiley et al. 2000, Wren, Seal et al. 2001). In this study, we identified a milk whey-derived hydrolysate with intrinsic ghrelin receptor agonist activity. The dairy hydrolysate, FHI-2571, dose-dependently and specifically increased intracellular $Ca^{2+}$ in HEK293A cells heterologously expressing the ghrelin receptor *in vitro*, while the unfractionated parent whey elicited negligible effects on the receptor (Figure 3 1A). *In vivo*, the ghrelin receptor is present throughout the small and large intestine, acting remotely via the vagus nerve to communicate with appetite centres in the brain (Date, Kojima et al. 2000, Date 2012). Given the appropriate oral delivery mechanism, a potential to increase food intake *in vivo* by targeting intestinal ghrelin receptor therefore exists.

Given the acidic and peptidase rich environment of the gastrointestinal tract, as well as the barriers to epithelial absorption, development of appropriate delivery platforms to improve *in vivo* efficacy of bioactive peptides is required (Brayden and Alonso 2016, Gleeson, Ryan et al. 2016). Microspheres and microcapsules are one such approach, however degradation of peptide due to complex processing steps is a concern (Witschi and Doelker 1998, Yin, Lu et al. 2008). Furthermore, there is an ever-increasing interest in nano-sized formulations (Date, Hanes et al. 2016). Nano-based approaches offer a platform to traverse membrane barriers and deliver peptide drugs in a targeted manner, increasing oral bioavailability and favourably altering

pharmacokinetic profiles (Griffin, Guo et al. 2016). However, much work remains to be done in order to elucidate the mechanisms of action and safety profiles of nano-formulations. Critically, despite the exciting advances in the micro- and nano- fields, none are yet proven as a viable, industrially scalable delivery approach to achieve both high loading of peptide, and a predictable release pattern. On the other hand, there are limited examples of conventional mm-sized pellets being used to deliver peptide payloads, despite the approach being widely used in formulation of small organic drug molecules. This is traditionally due to the poor permeation of peptides across the intestinal barrier, extensive first-pass metabolism and short half-life in the body, not to mention the high concentration of peptidases present in the upper small intestine. In the case of the bioactive peptide under investigation here, FHI-2571, its pharmacological target, the ghrelin receptor, is found throughout the small and large intestine on vagal afferent terminals located just beyond the mucosal brush border (Date, Kojima et al. 2000), while a substantial proportion of the hydrolysate size fraction is < 1 kDa, meaning that paracellular transit to these nerve terminals is possible (Griffin and O'Driscoll 2011). The ghrelin receptor is also located in the myenteric plexus of rodent and human gastrointestinal tract (Takeshita, Matsuura et al. 2006), furthering the case for enhancing the delivery of the peptidic payload to the intestinal lumen. Therefore, we sought to develop a simple, high loading sustained release delivery vehicle to protect the ghrelinergic peptide from acid exposure in the gastric compartment and upper small intestinal breakdown, to facilitate *in vivo* proof-of-concept studies.

Firstly, the need for a gastro-protected delivery vehicle was validated by exposing FHI-2571 to acidic pH, which predictably abolished the bioactivity of this compound on the ghrelin receptor in a progressive manner (Figure 3.2). Polymeric film coating of active pharmaceutical ingredient (API)-loaded core pellets has been widely utilized, with predominantly aqueous based functional polymer coatings (Siepmann and Siepmann 2013). EC is the most commonly-used coating polymer. It is non-toxic and biodegradable, and achieves predictable, pH independent release profiles due to drug diffusion across a water-insoluble membrane (Ozturk, Ozturk et al. 1990). Generally, aqueous EC colloidal dispersions are preferred as a coating medium due to safety and environmental reasons (Muschert, Siepmann et al. 2009,

Srivastava and Mishra 2010). Moreover, it is possible to achieve higher percentages of solid content in aqueous dispersions; the high viscosity of organic solutions of EC is a limiting step for coating media (Lecomte, Siepmann et al. 2004). Therefore, coating time can be excessively lengthened when organic solutions are used.

In our study, aqueous-based EC dispersion failed to provide us with a sufficiently robust film coating during release testing. Burst release of peptide was observed (> 80 % in the first 60 minutes) in USP Type-1 and USP-Type 4 apparatus. Macroscopic and microscopic investigation showed that the polymer coating fractured after exposure to aqueous media, allowing peptide to freely-diffuse out of the matrix system though fluid-filled cracks, rather than diffusing through the polymeric coat. This was attributed to the mechanism of film coat formation for a coating dispersion. During the fluid bed coating process, evaporation of solvent on the surface of the particles leads to sequential, layered polymer chain packing. These discrete polymer particles interact with one another via relatively weak Van der Waals interactions. Aqueous solubility is a major factor affecting osmotic pressure within coated pellets in contact with dissolution media. Osmotic pressure is a driving force for water ingress into pellets, increasing the intra-particulate volume and outward pressure on the coating film. Furthermore, migration of API into the film coat during the fluid-bed coating process has also been reported (Melegari, Bertoni et al. 2016). Therefore, the high aqueous solubility of the peptide may lead to leaching into the EC coat, creating water soluble pores which affords easier ingress of water into the pellet core, thereby causing swelling and an increased intra-particulate pressure. Considering the high loading of peptide in our system (33%), this problem is compounded leading to pellet swelling and film fracture.

Here, we demonstrate the mechanical integrity of two alternative film coating approaches, which both provide time-dependent release of a bioactive peptide in the *in vitro* setting. This is particularly useful in the context of sustained delivery of hydrophilic peptides. Initial burst release has been reported from some reservoir devices, which tapers over time due to a reducing concentration in the reservoir (Dekyndt, Verin et al. 2015). Organic solutions of the film coating polymer lead to greater mechanical strength in the resulting coat. This is due to the fluid movement of

the polymer chains in solution, which, upon removal of the solvent phase, cross-link and form a robust, physically-bonded polymeric meshwork (Lecomte, Siepmann et al. 2004). This is supported by SEM images (Figure 3.6) which show a distinct porous nature to the cross sectioned aqueous-based film coat (Figure 3.6B), compared to a more complete, non-permeable structure seen in the ethanolic-based film coat (Figure 3.6D). Consistent with the impervious nature of the coat show in SEM cross-sections, FHI-2571 -loaded pellets coated with an ethanolic solution of EC displayed near-zero order release in both USP Type 1 (Basket) and USP Type 4 (Flow-through) dissolution setups (Figures 3.4 & 3.7).

Due to the drawbacks associated with organic-based coating solutions, an aqueous -based coating approach to achieving an appropriate release profile of active peptide was desirable. Increased efficiency of aqueous EC film coats has been demonstrated by allowing a curing step to take place post-encapsulation, which typically involves extended periods of exposing the product to high temperature and humidity – water is an efficient plasticizer for many polymers (Kucera, Felton et al. 2013, Siepmann and Siepmann 2013). However, in this study an extended period of exposure to such harsh conditions was not possible due to the probability of peptide hydrolysis. Furthermore, layered multi-particulates have been used successfully by Siepmann and colleagues to provide reliable zero-order release of water-soluble agents (Dekyndt, Verin et al. 2015). However, this involved incorporating the drug into the film coating layer itself. The potential to incorporate a peptide-based bioactive into such coating solutions, rather than the pellet matrix itself, is limited due to high risk of denaturation.

Dual coated pellets have been used before to increase the functionality of the outer coat and optimize release profiles. In this study, an acid resistant methacrylic acid (MA) co-polymer was proposed, which was layered beneath the aqueous EC coat in order to provide an impermeable seal-coat in acid conditions. This may be considered atypical, given that a pH dependent polymer would normally form the outer layer in dual-coated systems. The acid-resistant layer was initially trialled as an overcoat of the aqueous EC coating, which limited burst release in acid conditions. However, upon transition to intestinal pH conditions, immediate dissolution of the MA

overcoat occurred, with subsequent "shelling" of the EC subcoat (data not shown). This was due to the swelling of the pellet core combined with the mechanically brittle subcoat formed by the aqueous-based EC dispersion. USP-Type 1 dissolution studies for our dual-coated pellets in SGFsp display a near-zero order release profile comparable to organic EC pellets (Figure 3.4). This is consistent with the insolubility of methacrylic acid below pH 5.5, which likely prevented osmotic fluid ingress into the pellet core, and subsequent pellet swelling and film fracture. Furthermore, USP-Type 4 dissolution studies, also show a delayed release profile after transitioning to intestinal media (pH 6.8) (Figure 3.7). This may be considered surprising given the solubility of the methacrylate copolymer above pH ~ 5.5. The intact EC overcoat in this case is likely hindering the access of the intestinal buffer to the surface of the subcoat, thereby reducing the rate at which the subcoat can dissolve. The advantage to the MA applied as a subcoat is therefore two-fold, initially it prevents the ingress of fluid to the pellet core and subsequent pressure-induced film fracture. Secondly, the limited exposure of intestinal media to the MA subcoat due to the intact EC overcoat serves to slow the overall dissolution of the film coat.

BARDS was utilized to confirm the release profiles obtained from the compendial dissolution methods (Figure 3.8). This is an emerging technology used to explore the changes in compressibility of a solvent that occurs during dissolution. During an experiment, the introduction of the pellets into the BARDS system causes changes in the speed of sound in the dissolution medium, which can be monitored acoustically. The dissolution process thus generates a change in the resonance frequency time course of the solvent in the vessel. BARDS analysis has previously shown successful application in the analysis of powder blend uniformity (Fitzpatrick, Scanlon et al. 2012) and the profiling of enteric-coated drug delivery systems (Fitzpatrick, Evans-Hurson et al. 2014).

Of vital importance to this work was to confirm that the active peptide retains its bioactivity post-encapsulation, as protein aggregation or denaturation may occur during formulation. In fluid-bed coating, inlet air temperature is partially negated due to the latent heat of evaporation of the coating polymer solvent during the spraying process (El Mafadi, Picot et al. 2005, Poncelet D 2009). This leads to a milder micro-

temperature at the surface of individual pellets than would be suggested by the process parameters themselves. In our study, peptide liberated from the pellet formulation displays > 80 % activity of the untreated peptide. Compared to alternative methods of encapsulation which have been used for peptides incorporating solvent-based methods, we consider this to be a reasonable retention of activity in light of our processing conditions.

In conclusion, a multiparticulate sustained release formulation approach for delivery of a ghrelin agonist peptide is described. Aqueous-based EC film coats applied to pellets in the millimetre size range are porous and mechanically brittle, leading to disintegration or "shelling" of the coat in aqueous media. Here, we observed that the high loading of a freely soluble ghrelin agonist peptide enhanced the problem of film disintegration due to increased osmotic pressure and pellet swelling. To overcome this, we provide near zero-order release by taking two alternative approaches: 1) Organic EC based solution can be applied to the pellets, or 2) an aqueous dispersion of a pH dependent MA co-polymer may be introduced as a subcoat to the aqueous EC. This provides an impermeable seal coat in gastric conditions which prevents fluid ingress into pellets, thereby preventing pressure-induced EC layer fracture and allowing the EC polymer to function as originally intended. Both processes allow the ghrelinergic peptide to retain sufficient activity after encapsulation. In conclusion, we designed a successful delivery formulation for a peptide based ghrelinergic dairy-derived bioactive hydrolysate. This delivery platform is suitable for progression to pre-clinical rodent models to assess efficacy *in vivo*.

# Chapter 4

# Behavioural characterization of novel ghrelin ligands, Anamorelin and HM01: Appetite and reward-motivated effects in rodents

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# Abstract

The ghrelinergic system, comprising of the neuroendocrine peptide, ghrelin, and its receptor, the growth hormone secretagogue receptor (GHSR-1a), have been steadily investigated as therapeutic targets in the treatment of metabolic and appetite disorders. Despite nearly 2 decades of concerted efforts, the outcomes of this field of research have been disappointing. It is becoming increasingly clear that ghrelinergic signalling has both unexpected and unexploited complexities in its pharmacology, which have likely been hindering research efforts. While native ghrelin activates the full complement of GHSR-1a pathways, synthetic GHSR-1a ligands display biased signalling and functional selectivity, which have a significant impact on the intended and indeed, unintended, therapeutic effects. Furthermore, the widespread expression of the GHSR-1a *in vivo* has led to increasing consideration of the biodistribution of GHSR-1a ligands. Here, we investigate anamorelin and HM01, two novel synthetic GHSR-1a ligands with promising effects on food intake in preclinical and clinical studies. Downstream signalling pathways of both are compared in calcium mobilization, IP-one, internalization and β-arrestin recruitment assays. We describe a novel divergent activation of central reward circuitry by anamorelin and HM01 using c-Fos immunostaining as well as behavioural effects in food intake and reward paradigms.

Interestingly, we found a paradoxical reduction in reward-related behaviour for anamorelin and HM01 treated animals in our chosen paradigms. The work highlights the critical importance to consider signalling bias in relation to future ghrelin-based therapies. In addition, central access of GHSR-1a ligands, particularly to reward areas of the brain, remains a crucial factor in eliciting potent appetite-stimulating effects. The precise characterization of downstream ghrelinergic signalling and biodistribution of novel GHSR-1a ligands will be decisive in their successful development and will allow predictive modelling and design of future synthetic ligands to combat metabolic and appetite disorders involving the ghrelinergic system.

# Introduction

Since its discovery in 1999, efforts to exploit ghrelin's orexigenic capacity for disorders of appetite have been met with limited success. Endeavours in the appetite modulation field have found the growth hormone secretagogue receptor (GHSR-1a) to be an elusive target with a deceptively complicated pharmacological profile (Howick, Griffin et al. 2017). Ghrelin, the endogenous ligand for GHSR-1a, is a peripherally-produced endogenous hormone which acts centrally as a key mediator in the neuroendocrine control of food intake, metabolism and adiposity (Tschop, Smiley et al. 2000, Nakazato, Murakami et al. 2001). The primary site of production and release is the stomach (Kojima, Hosoda et al. 1999), with plasma levels of ghrelin peaking in anticipation of a meal, while the arcuate nucleus of the hypothalamus (Arc) is the main site of action for effecting an increase in food intake (Cummings, Purnell et al. 2001, Nakazato, Murakami et al. 2001, Cowley, Smith et al. 2003). The orexigenic effects of ghrelin are mediated via activation of its target G-protein coupled receptor, the GHSR-1a, which has been conclusively demonstrated across species (Wren, Small et al. 2000, Cummings, Purnell et al. 2001, Nagaya, Uematsu et al. 2001, Wren, Seal et al. 2001, Mericq, Cassorla et al. 2003, Chen, Trumbauer et al. 2004, Druce, Wren et al. 2005, Schmid, Held et al. 2005, Wynne, Giannitsopoulou et al. 2005). As such, the GHSR-1a represents a promising therapeutic target for conditions of under-eating such as Cancer Anorexia Cachexia Syndrome (CACS) (Nass, Gaylinn et al. 2011, Howick, Griffin et al. 2017), as well as over-eating and obesity (Soares, Roncon-Albuquerque et al. 2008, Schellekens, Dinan et al. 2010). Treatment with ghrelin has shown promising results on food intake and lean body mass maintenance in preclinical animal models of CACS, as well as clinically (Nagaya, Uematsu et al. 2001, Wynne, Giannitsopoulou et al. 2005). However, the short half-life and ready deactivation *in vivo* into des-acylated ghrelin means that the pharmacokinetics of the ghrelin peptide are not optimal to provide sustained increases in appetite (Delhanty, Neggers et al. 2012, Delhanty, Neggers et al. 2014).

Numerous synthetic ghrelin ligands have been developed over the years with the aim of providing sustained, desirable alterations in appetite (Vodnik, Štrukelj et al. 2016). However, to date few have reached the market, likely due to the increasingly

recognized complexity in GHSR-1a pharmacology (Howick, Griffin et al. 2017, Ramirez, van Oeffelen et al. 2018). GHSR-1a elicits various downstream signalling pathways which are ligand-dependent, while also exhibiting a high degree of basal, ligand-independent activity (Ramirez, van Oeffelen et al. 2018). Largely ignored until recently, these differences in the functional selectivity of ghrelin ligands can have an impact on the ultimate effect observed *in vivo* (M'Kadmi, Leyris et al. 2015, Mende, Hundahl et al. 2018). There is growing evidence that selectively activating GHSR-1a signalling with pathway-specific ligands may lead to the development of more successful candidates to treat appetite disorders, while minimising off-target effects. Recent literature has described the importance of biased ligand signalling. Importantly, it has already been shown that $G_q$ blockade specifically is responsible for eliciting a decrease in food intake (Mende, Hundahl et al. 2018). Thus, there is a growing impetus for characterisation of the signalling pathway(s) activated by individual ligands, and their subsequent contribution to the observed behavioural effect.

Hence, given the lack of a successful ghrelin-based therapeutic to date, an appreciation of the pleiotropic pharmacodynamics of the GHSR-1a is crucial. The biodistribution of ghrelin ligands also has a significant role to play in determining *in vivo* effects (Howick, Griffin et al. 2017). While the hypothalamus is the traditional site of action for food intake and body weight regulation, the GHSR-1a is also expressed in key nodes of the reward system and contributes to so-called "pleasurable" eating beyond metabolic demand (Abizaid, Liu et al. 2006, Zigman, Jones et al. 2006). Indeed, ghrelin treatment has been shown to increase the motivation to work for a food reward in rodents, as well as shifting the preference from standard chow towards palatable, calorie-dense foods (Shimbara, Mondal et al. 2004, Egecioglu, Jerlhag et al. 2010, Skibicka, Hansson et al. 2011). Ghrelin's ability to effect this despite a lack of apparent ability to gain access to the brain is a source of ongoing debate in the field (Cabral, De Francesco et al. 2015, Edwards and Abizaid 2017). Therefore, the biodistribution of the GHSR-1a in areas not immediately accessible to the peripheral circulation has given rise to the theory that central penetrance of ghrelin ligands would be advantageous, for example by increasing the access of ghrelin ligands to the mesolimbic reward circuitry (Howick, Griffin et al. 2017).

More detailed mechanistic research is required to inform the pharmacodynamics and biodistribution of ghrelin ligands, in order to fully elucidate their therapeutic merit in disorders of appetite. This paper provides *in vitro* and *in vivo* characterization of two novel, synthetic GHSR-1a agonists, anamorelin (non-BBB penetrant) and HM01 (BBB penetrant), previously demonstrated to exhibit a high GHSR-1a potency and selectivity, good oral bioavailability and longer half-lives than ghrelin (approximately 7 and 4.5 hours respectively). Both have already shown promising results on food intake and lean body mass maintenance in preclinical animal models of cachexia (Pietra, Takeda et al. 2014, Borner, Loi et al. 2016). Here, we characterize and compare the signalling pathways of anamorelin and HM01 to native ghrelin on the GHSR-1a *in vitro.* Furthermore, the divergent neuronal activation underlying the ligands is explored using c-Fos immunohistochemistry, while effects on appetite and reward-motivated behaviour is also assessed. Knowledge of the downstream signalling pathways of GHSR-1a, and an appreciation of the role of GHSR-1a in the reward system is crucial to predicting the effect observed *in vivo.* Taken together, this paper provides novel insights into key factors, which are poised to pave the road to success for future ghrelinergic therapies.

# Materials and Methods

## 4.1 Cell lines and reagents

Fetal bovine serum (3.3%) was purchased from Sigma-Aldrich, Arklow, Wicklow, F7524. Assay buffer consisted of 1x Hanks balanced salt solution, HBSS, Gibco™ 14065049 (Thermo Fisher Scientific™), supplemented with 20 mM HEPES (Sigma-Aldrich, Arklow, Wicklow, H0887). The endogenous agonist, ghrelin (rat), was obtained from Tocris Bioscience, Avonmouth, Bristol, UK (Cat. No. 1465). Synthetic ghrelin agonists HM01 and Anamorelin were kindly provided by Helsinn Therapeutics, Lugano, Switzerland.

## 4.2 *In vitro* assays for GHSR-1a mediated signalling

### 4.2.1 $Ca^{2+}$ mobilization assay

This method has been described in detail in Section 3.3 above.

### 4.2.2 IP-one mobilization assay

The detection of IP-one was performed in HEK293A cells expressing GHSR-1a, according to the manual's instruction from Cisbio (Codolet, France). Briefly, 24 hours before experiment, growth media was replaced with serum free DMEM containing 1% NEAA. Directly before the experiment cells were manually disrupted by scraping in PBS and centrifuged for 3 minutes at 200 x g. A cell pellet was then suspended in assay buffer (146 mM NaCl, 1 mM $CaCl_2$, 10mM HEPES, 0.5 mM $MgCl_2$, 4.2 mM KCl, 5.5 mM glucose) containing 50 mM LiCl. For the stimulation step, 35 µL of cell suspension was pipetted into a flat bottom 96-well plate at the density of 3 x $10^5$/well containing the appropriate compound solution, and incubated for 30 minutes at 37 °C. Following this, 15 µL of IP1-d2 conjugate and 15 µL of anti-IP1 cryptate conjugate in lysis buffer were added and incubated for 1 h in room temperature, followed by fluorescent measurement. After 1 h of incubation at room temperature, the fluorescence at 620 nm and 665 nm was read with the use of FlexStation (Molecular Devices, LLC Sunnyvale, CA). The results were calculated as the 665-nm/620-nm

ratio multiplied by $10^4$ and depicted as percentage of relative fluorescent units (RFU) normalized to maximum response (100% signal) obtained for non-stimulated cells. This was then converted to demonstrate the proportional dependence of the signal to the level of endogenous IP-one in the sample.

### 4.2.3 Internalization assay

Ligand-mediated GHS-R1a receptor translocation was quantified by monitoring the EGFP fluorescent trafficking away from the cellular membrane into vesicles within the cytosol, as per a previously described protocol (Torres-Fuentes, Pastor-Cavada et al. 2018). Cells were seeded in 96-well plate at density of 2.5 x $10^4$ cells/well and incubated for 48 hours at standard culture conditions. 24 hours before the experiment, media was replaced with serum free DMEM containing 1% NEAA. Cells were incubated with different concentrations of GHSR-1a receptor ligands for 60 minutes in 37ºC. After treatment, cells were fixed with 4% paraformaldehyde in phosphate buffer saline (PBS) for 20 minutes, and washed two times with PBS. Cells were imaged on the GE Healthcare IN Cell Analyzer 1000 (GE Healthcare Life Science, Buckinghamshire UK) and receptor trafficking analysed using the IN Cell Analyzer Developer Toolbox V1.6 Software (GE Healthcare). The intracellular EGFP intensity increase was normalized to Buffer B.

### 4.2.4 Beta-arrestin recruitment assay

PathHunter® eXpress GHSR-1a U2OS β-Arrestin-1 GPCR Assay (93-0242E3CP5S, Discoverx, Fremont, CA) was used to analyse the effects of GHS-R1a receptor ligands on both basal and agonist-mediated β-Arrestin-1 recruitment. Procedures were performed according to the manufacturer's instructions. Briefly, cryopreserved PathHunter® eXpress GHSR-1a U2OS cells were plated at a density of 2.5 x $10^3$ cells/well of the 96-well plate. After 48 hours incubation at standard culture conditions, cells were treated with GHS-R1a receptor ligands for 60 minutes. Luminescent signal was read with the use of Synergy 2 (Biotek Instruments, Winooski, VT).

### 4.2.5 Animals

Male Sprague-Dawley rats (8 weeks) and C57Bl/6 mice (8 weeks) were purchased from Envigo, United Kingdom for use in *in vivo* behavioural experiments. All animals were housed in group cages at 21 ± 1°C, humidity (55± 15 %), outside air ventilation (15±5 cycles/h) with a 12-h light/dark cycle. Animals were acclimatized for at least 1 week before use in experiments. Animals were provided standard chow (Teklad Global 18 % Protein Rodent Diet, Envigo, UK) and tap water *ad libitum*. All experiments were performed in accordance with European guidelines following approval by University College Cork Animal Ethics Experimentation Committee (B100/3774).

### 4.2.6 *Ex vivo* c-Fos immunohistochemical analysis

Rats were randomly allocated to one of four treatment groups (saline, ghrelin 0.3mg/kg, anamorelin 3mg/kg or HM01 3mg/kg)(Wren, Small et al. 2001, Pietra, Takeda et al. 2014, Naitou, Mamerto et al. 2015). On the morning of experiment, animals were administered with a single IP injection of the relevant compound and individually housed and left undisturbed for a period of 2 hours, after which a lethal dose of anaesthetic was administered, and the animals perfused with chilled phosphate-buffered saline followed by 0.4% paraformaldehyde (PFS) fixative. Brains were removed and stored in a 0.4% PFA for 24 hours, after which they were transferred to a 30% sucrose solution for a period of 1 week. Brains were then snap frozen in liquid nitrogen and stored at -80C until sectioning. Sections from the requisite brain areas (20 µm) were cut in a Leica cryostat (model CM100), thaw-mounted on microscopic glass slides and stored at -80 C until further processing. AP coordinates were verified microscopically for replicate slices using the stereotaxic atlas. Before staining, sections were rehydrated in Phosphate Buffered Saline (PBS; 0.01M) for 5 minutes. Endogenous peroxidase activity was removed by immersing the sections in a 0.24% $H_2O_2$. Slides were washed in PBS containing 0.2% Triton-X-100 (PBS-T) twice for 5 minutes each. To block unspecific binding, slides were incubated in blocking solution (PBS containing 0.2% Triton R X-100 and 3% normal goat serum (GS) for 1 hour at room temperature. The primary antibody (rabbit monoclonal anti-

c-Fos (#2250, Cell Signalling Technology, 1:10,000 in PBS) containing 0.2% Triton R X-100 and 3% GS solution was applied for 24 h at 4C. The secondary antibody (biotinylated goat-anti rabbit, Vector Laboratories, 1:200 in PBS containing 0.2% Triton R X-100) was applied for 2 hours at room temperature. Sections were then incubated in avidin-biotin complex (ABC) reagent using a kit for 90 minutes (ABC Vectastain R , Burlingame, CA, USA) for 1 h at room temperature. After this, sections were washed in PBS-T and incubated with the chromogen (0.5mg/ml Diaminobenzidine, DAB) for up to 5 minutes, or until a brown colour started to develop. Sections were counterstained with cresyl violet for 10 minutes and subsequently dehydrated in a series of alcohol solutions before clearing in histolene and mounting of DPX. c-Fos positive cell counts were averaged per animal from a defined frame size from at least three slices (maximum of five slices) and used for the calculation of group means.Photomicrographs were taken at 20x magnification, using a digital camera system (Olympus BX53 upright microscope, Olympus Life Science).

## 4.3 Behavioural Experiments

### 4.3.1 Cumulative Food Intake

All animals were habituated to single housing conditions and procedures for up to 5 days prior to experimental day. Rats were randomly allocated to one of four treatment groups (Saline, Ghrelin 0.3mg/kg, Anamorelin 3mg/kg or HM01 3mg/kg). On the morning of experiment, animals were individually housed for 30 minutes, after which they were administered with a single IP injection of the relevant compound. Thereafter, food intake was monitored hourly for a period of 7 hours by quantifying the amount of leftover food. The amount of food consumed at 24 hours post-dose was also recorded. Cumulative change in food intake, as well as an hourly breakdown of the time course of food intake was evaluated.

### 4.3.2 Saccharin Preference Test

Rats were individually housed with *ad libitum* access to standard chow and randomly allocated to one of three treatment groups (saline, anamorelin 3mg/kg or

HM01 3mg/kg). Each rat was habituated to two water bottles in the cage for up to 8 hours a day over 4 days to familiarize the rats to drinking from two bottles. During training, one bottle contained water while the other contained a 0.1% saccharin solution, a concentration shown in the literature studies to provide a robust but not maximal saccharin preference (Sclafani, Bahrani et al. 2010). During the habituation phase, the bottles were alternated in order to prevent a side-bias from confounding results. Bottles were weighed before and after each habituation session to monitor for a preference establishment. On the experimental day, animals placed into individual cages and injected IP at the onset of the light phase. *Ad libitum* access to water, 0.1% saccharin solution and standard chow was available throughout the experiment. Consumption of/preference for saccharin was monitored over a 24 hour period.

### 4.3.3 Female Urine Sniffing Test

The protocol for assessing female urine sniffing behaviour in male C57Bl/6 mice was carried out as per Malkesman et. al (Malkesman, Scattoni et al. 2010). Mice were randomly allocated to one of three treatment groups (saline, ghrelin, anamorelin 3mg/kg or HM01 3mg/kg). One week before the test, mice were placed into individual cages in order to remove the effect of single housing on the day of the experiment. On the experimental day, rodents were transferred to a dark room illuminated with a red-light. 1 hour before the test, mice were habituated to the presence of a cotton-tipped applicator extending into the home cage. Then, 30 minutes before the test mice were given an intraperitoneal (IP) injection with the appropriate treatment or saline control. The following protocol took place for each mouse; a 3 minute exposure to a cotton tip dipped in 60µL sterile water, during which the experimenter left the room and video was recorded for later analysis of duration of interaction, total number of interactions and latency to interact. This was followed by an inter-trial interval of 45 minutes during which no cotton tip was in the cage. Lastly, a 3 minute exposure to a cotton tip dipped in 60µL of urine, freshly collected from a cohort of female mice in estrous, was performed, during which the same parameters were recorded.

## 4.4 Data Analysis

Data were analysed and graphs generated using both GraphPad Prism software and Microsoft Excel software. All means were calculated from the results of at least three independent experiments carried out in triplicate. For the *in vitro* calcium mobilization assays, standard error of the mean (SEM) is depicted, while all dissolution results report standard deviation (SD). For the c-Fos immunostaining, a Kruskal-Wallis analysis with Dunn's post-hoc test for multiple comparisons was used to determine statistical significance. A repeated measures ANOVA with Tukey's post-hoc test for multiple comparisons was used to determine significance in the food intake, SPT and FUST paradigms.

# Results

## 4.5 Potency and efficacy profiles of Anamorelin and HM01

The GHSR-1a exerts ligand-dependent biased signalling and upon activation can send downstream signalling via Gαq- dependent signalling, which is critical for food intake behaviour (Mende, Hundahl et al. 2018). The agonist activity of HM01 and anamorelin on the GHSR-1a was measured using an intracellular $Ca^{2+}$ mobilization assay, as a measure of downstream GHSR-1a signalling in HEK293A cells (human embryonic kidney cells) stably expressing the GHSR-1a tagged with an enhanced green fluorescent protein (GHSR-1a-EGFP) (Figure 4.1) (Schellekens, van Oeffelen et al. 2013),. Interestingly, both HM01 ($EC_{50}$ = 8.8 x$10^{-10}$ M) and anamorelin ($EC_{50}$ = 1.1x$10^{-8}$ M) display higher potencies compared to the endogenous receptor ligand, ghrelin ($EC_{50}$ = 4.5x$10^{-8}$ M). In addition, the maximal response attained for both ligands (HM01 $E_{max}$ = 117%, Anamorelin $E_{max}$ = 129%) is the same as compared to ghrelin ($E_{max}$ = 127%).

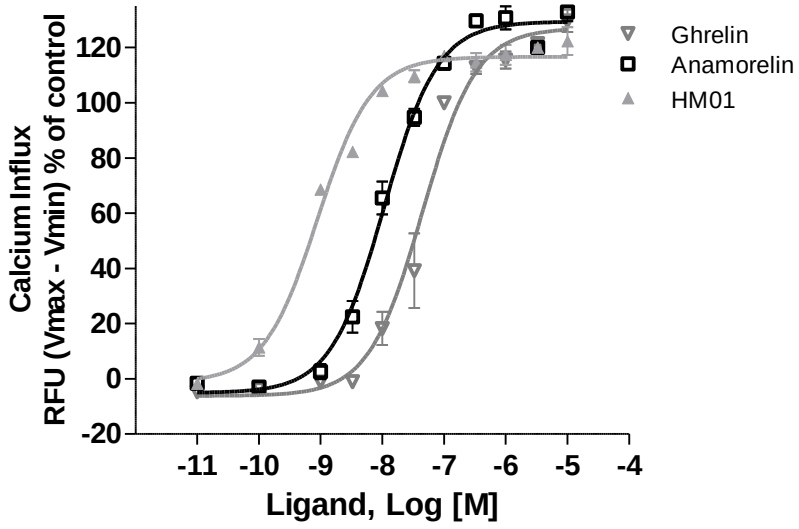



***Figure 4.1. Concentration response curve of novel GHSR-1a ligands****. Concentration response curves for the endogenous GHSR-1a ligand, ghrelin, and the synthetic GHSR-1a ligands, HM01 and anamorelin measured in HEK293A cells stably expressing the GHSR-1a. Intracellular $Ca^{2+}$ increase was depicted as a percentage of*

*maximal $Ca^{2+}$influx in relative fluorescence unit (RFU) as elicited by control (3.3% FBS). Graph represents mean ± SEM of at least three independent experiments performed in triplicate.*

## 4.6 IP-one mobilization assay

Next, the inositol-phosphate one (IP-one) assay was carried out to confirm the efficacy of the synthetic ligands on the Gαq signalling-pathway as seen with the $Ca^{2+}$ mobilization assay. Again, anamorelin and HM01 produced a stronger concentration-dependent agonist effect compared to ghrelin. The potencies of both HM01 ($EC_{50}$ = 2.3 $x10^{-10}$ M), and anamorelin ($EC_{50}$ = 5.2 $x10^{-9}$ M) were higher compared to that obtained with ghrelin ($EC_{50}$ = 3.1 $x10^{-8}$ M). Interestingly, the maximal response attained for both ligands (HM01 $E_{max}$ = 106%, anamorelin $E_{max}$ = 111%) is the same as compared to ghrelin ($E_{max}$ = 111%).

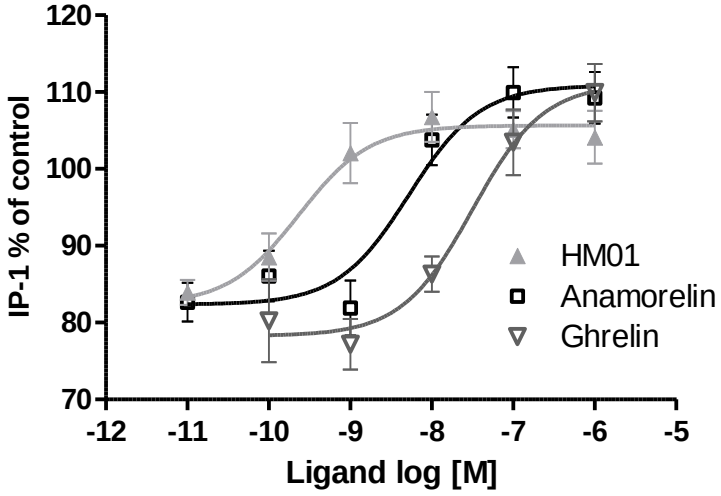



***Figure 4.2. Inositol phosphate one (IP-one) accumulation after treatment with ghrelin and novel ghrelin ligands.*** *Fluorescence intensity (IP-one accumulation) increases in a concentration-dependant manner for the endogenous GHSR-1a ligand, ghrelin, and the synthetic GHSR-1a ligands, HM01 and anamorelin measured in GHSR-1a expressing HEK293A cells. Intracellular IP-one accumulation was depicted as a percentage of maximal $Ca^{2+}$influx in relative fluorescence unit (RFU) as elicited by control (3.3% FBS). Graph represents mean ± SEM of at least three independent experiments performed in triplicate.*

## 4.7 Internalization assay

Next, the effects of anamorelin and HM01 on GHSR-1a internalization into

endosomal vehicles were evaluated. Desensitization and internalization provide a pivotal feedback loop preventing overstimulation through the GHSR-1a (Ramirez, van Oeffelen et al. 2018). Clear GHSR-1a internalization was observed after treatment with ghrelin, anamorelin and HM01. The internalization was dependent on the concentration of ligand used; Ghrelin $EC_{50}$ = 5.3 x $10^{-9}$ M, anamorelin $EC_{50}$ = 2.7 x $10^{-8}$ M, HM01 $EC_{50}$ = 2.3 x $10^{-10}$ M. The pattern of potencies is aligned with those reported above, but interestingly the $E_{max}$ reached by anamorelin (126%) is much higher than that of ghrelin (74%) and HM01 (69%) as a percentage of control (3.3% FBS) (Figure 4.3).

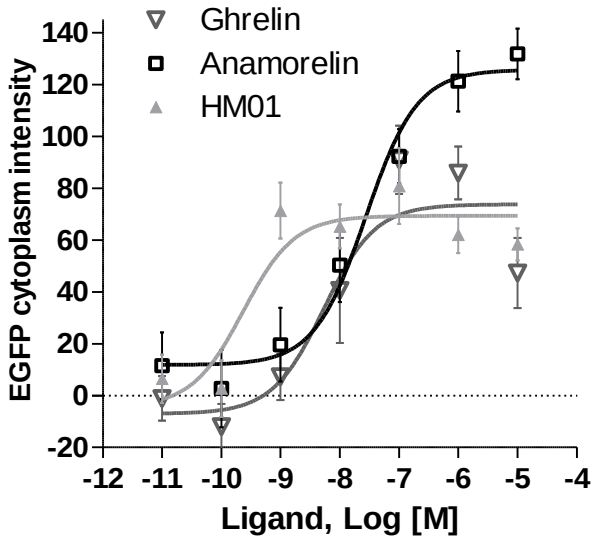



***Figure 4.3. Internalization of GHSR-1a after treatment with ghrelin or novel GHSR-1a ligands.*** *Cytoplasmic EGFP intensity, as a measure of the GHSR-1a-EGFP internalization, increases in a concentration-dependant manner for ghrelin as well as for the synthetic GHSR-1a ligands, HM01 and anamorelin, measured after a 1 hour incubation period in HEK293A-GHSR-1a cells. Graph represents mean ± SEM of at least three independent experiments performed in triplicate.*

## 4.8 β-arrestin recruitment assay

The intracellular protein, β-arrestin, functions in the desensitization of GPCRs and in the control of their intracellular trafficking(Bologna, Teoh et al. 2017, Ramirez, van Oeffelen et al. 2018). Here, β-arrestin recruitment was assessed after pre-treatment with ghrelin, anamorelin and HM01, to further support the findings of the ligand-

mediated GHSR-1a internalization, as reported above. As expected, ghrelin ($EC_{50}$ = 2.3 x $10^{-8}$ M), anamorelin ($EC_{50}$ = 1.9 x $10^{-8}$ M) and HM01 ($EC_{50}$ = 2.3 x $10^{-9}$ M) increase the recruitment of β-arrestin in a concentration dependant manner. Critically this result aligns with the previously observed ligand-mediated GHSR-1a internalization results as the $E_{max}$ reached by anamorelin (153%) is again much higher than that of ghrelin (107%) and HM01 (89%).

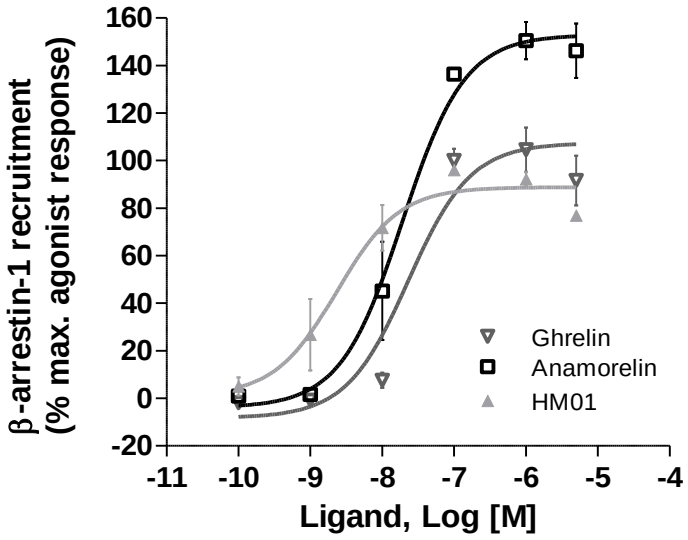


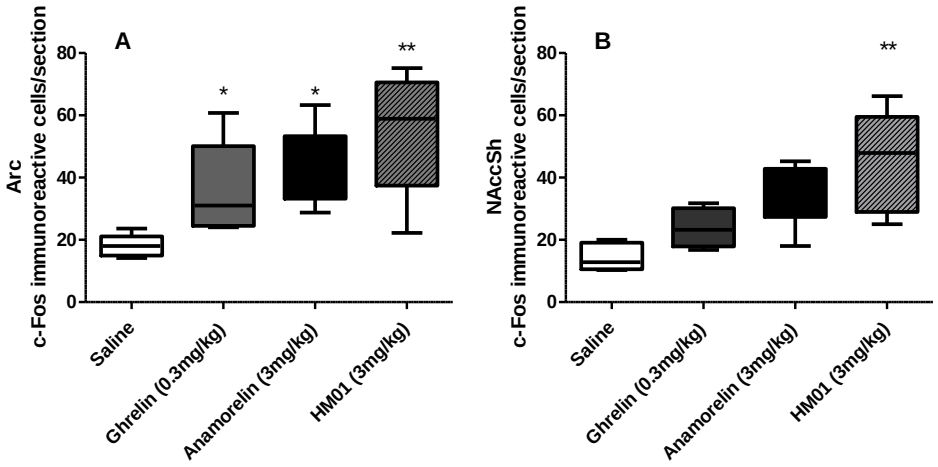

***Figure 4.4. Recruitment of β-arrestin after treatment with ghrelin or novel GHSR-1a ligands*** *Luminescent signal intensity (β-arrestin recruitment) increases in a concentration-dependant manner for ghrelin and the synthetic GHSR-1a ligands, HM01 and anamorelin measured in HEK293A-GHSR1a cells. The β-arrestin recruitment was depicted as a percentage of maximal agonist response as elicited by control (3.3% FBS). Graph represents mean ± SEM of three independent experiments performed in triplicate.*

***Table 4.1. Summary table of potency and efficacy of ghrelin, anamorelin and HM01 in vitro***

| | ***Potency (EC50) [M]*** | | | ***Efficacy (Emax) %*** | | |
|---|---|---|---|---|---|---|
| ***Assay*** | ***Ghrelin*** | ***Anamorelin*** | ***HM01*** | ***Ghrelin*** | ***Anamorelin*** | ***HM01*** |
| *$Ca^{2+}$ mobilization* | *$4.5x10^{-8}$* | *$1.1x10^{-8}$* | *$8.8x10^{-10}$* | *127.1* | *129.4* | *116.6* |
| *IP-one assay* | *$3.2x10^{-8}$* | *$5.2x10^{9}$* | *$2.3x10^{-10}$* | *111.1* | *110.9* | *105.7* |
| *Internalization* | *$5.3x10^{-9}$* | *$2.7x10^{-8}$* | *$2.3x10^{-10}$* | *69.3* | *125.7* | *73.7* |
| *B-arrestin* | *$2.3x10^{-8}$* | *$1.9x10^{-8}$* | *$2.3x10^{-9}$* | *107.34* | *152.9* | *88.8* |

## 4.9c-Fos immunohistochemistry

Next, neuronal activation was quantified using c-Fos immunohistochemical staining. Predictably animals treated with ghrelin, anamorelin and HM01 demonstrated a significant elevation in arcuate neuronal activation compared to saline vehicle. Interestingly, divergent activation profiles were obtained for HM01 vs. anamorelin; a significant increase in immunoreactivity was noted in the LH, VTA and NAccSh for HM01, all of which are key areas in the reward pathway which are not peripherally accessible. Kruskal-Wallis with Dunn's post-hoc test for multiple comparisons was used to determine statistical significance ((A):Kruskal-Wallis (KW) statistic =12.04, p=0.0072, (B) KW =13.14, p=0.0043, (C) KW = 8.046, p=0.0451, (D) KW=11.67, p=0.0086). The spatial separation of the peripherally active anamorelin is a limiting factor to the activation of brain areas not immediately accessible to the peripheral circulation. This strongly indicates the importance of biodistribution of GHSR-1a ligands in the prediction of functional outcome *in vivo.*



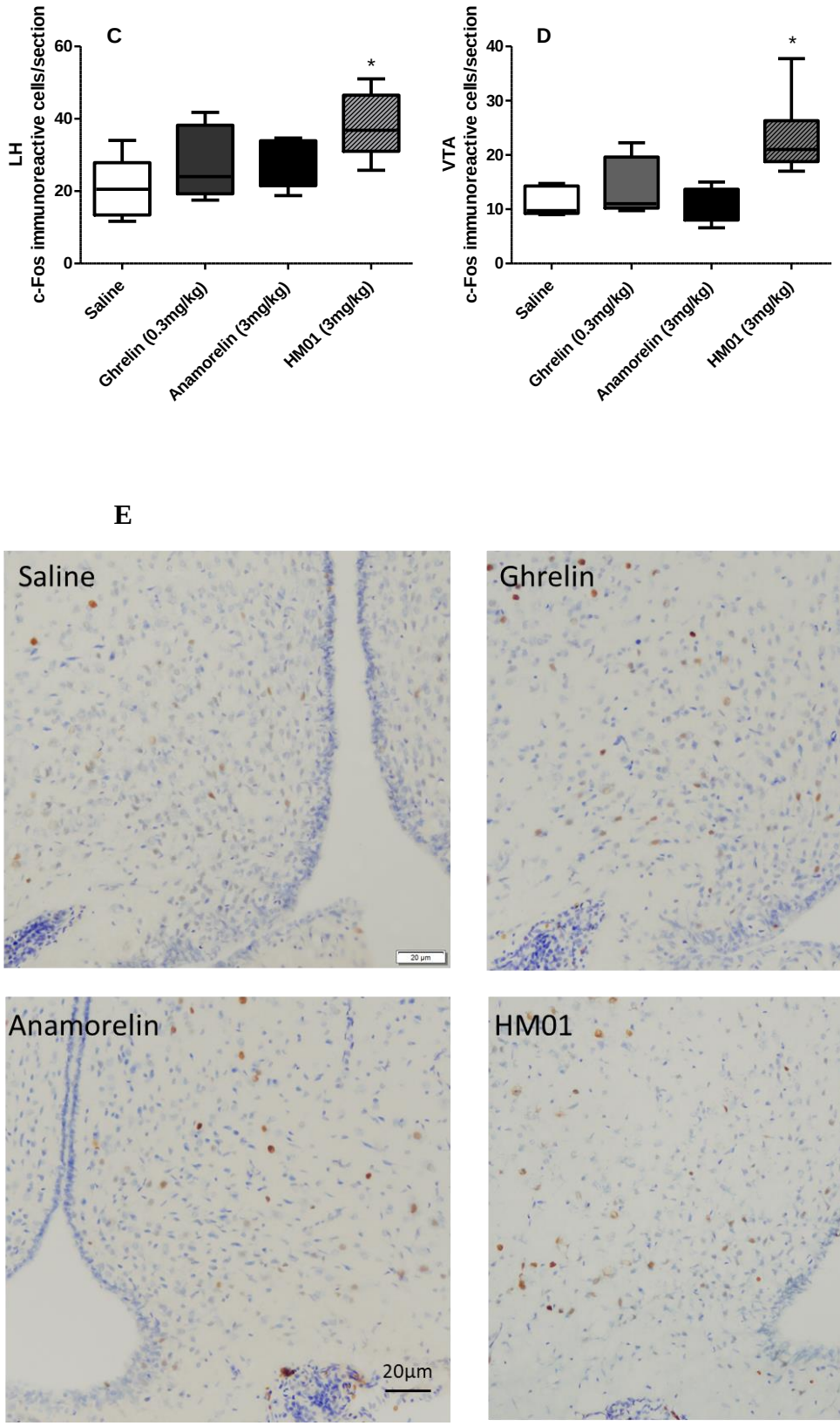
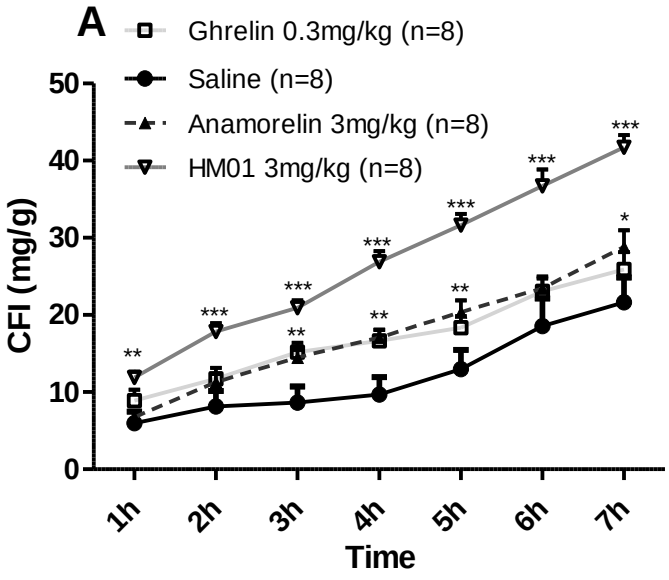



***Figure 4.5. Effect of ghrelin, HM01 and anamorelin on c-Fos expression in homeostatic and reward centres.*** *c-Fos expression levels were quantified in Arc (A), LH (C), VTA (D) and NAccSh (B) following intraperitoneal (IP) injection with*

*0.3mg/kg body weight of ghrelin, or 3mg/kg of anamorelin or HM01. Representative images of arcuate nucleus staining for c-Fos neuronal activation at 20x magnification (E). Kruskal-Wallis with Dunn's post-hoc test for multiple comparisons was used to determine statistical significance; depicted as **p < 0.01 and *p < 0.05.*

## 4.10 Cumulative Food Intake

*In vivo* effects of the novel ligands were assessed in an acute food intake paradigm. Since manipulation of the ghrelinergic system is known to stimulate food intake, the amount of standard rodent chow consumed was monitored over a 24-hour period after administration of a ghrelin ligand or control (Figure 4.6). Repeated measures analysis revealed an overall effect of time (p=<0.001, df =1, F=289.081), treatment*time (p=<0.001, df = 3, F=257.615) and treatment (p=<0.001, df=3, F=19.623). Tukey's post hoc test for multiple comparisons showed a significant effect of ghrelin (p=0.018), Anamorelin (p=0.012) and HM01 (p<0.001) compared to vehicle. Interestingly, multiple comparisons revealed there was a statistically significant effect observed between HM01 and both ghrelin (p= <0.001) and anamorelin (p=<0.001). Analysis of cumulative food intake 24 hours post-dose showed an overall effect of treatment (p=0.018, df=3, F=3.943). The orexigenic effect of HM01 was sustained at the 24-hour timepoint, while anamorelin maintains a trend (p=0.061). Ghrelin's orexigenic effect is not sustained after 24 hours, in agreeance with previous studies (Finger, Schellekens et al. 2011, Schellekens, De Francesco et al. 2015). Post-hoc analysis showed that ghrelin's effect tapered after 4 hours.



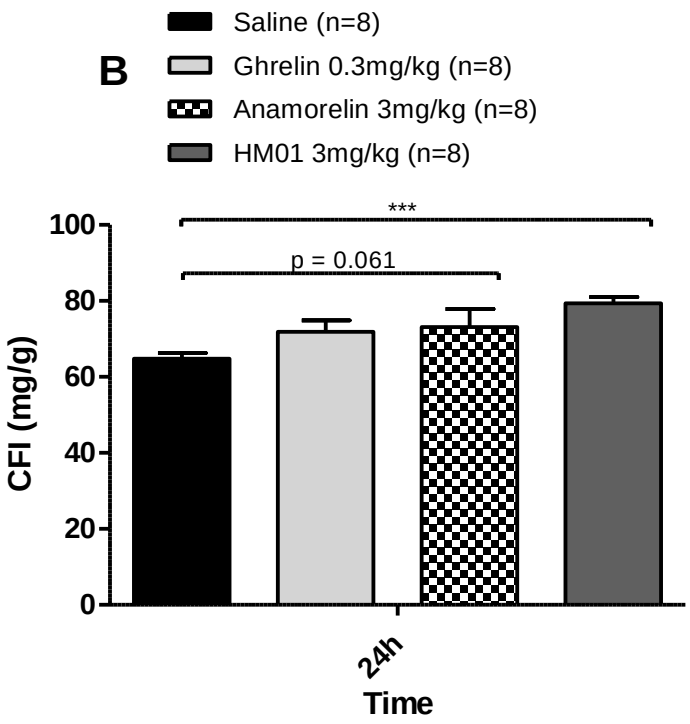
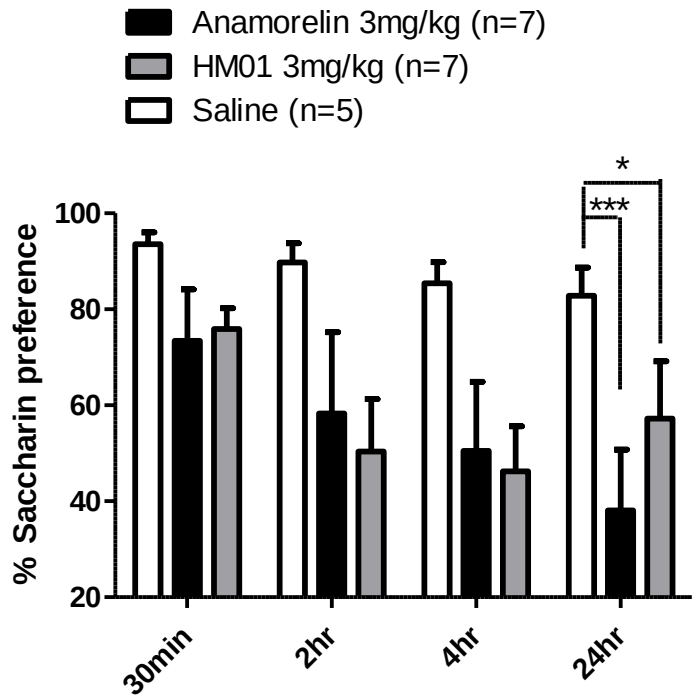



***Figure 4.6. Cumulative food intake following intraperitoneal administration of ghrelin, HM01 and anamorelin.*** *Food (regular chow) intake in male sprague-dawley rats was determined following intraperitoneal (IP) injection with 0.3mg/kg body weight of ghrelin, or 3mg/kg of anamorelin or HM01 over 7 hours. Cumulative food intake (CFI) was determined at regular intervals after dosing. There was an overall effect of time (p=<0.001, df =1, F=289.081) and treatment*time (p=<0.001, df = 3, F=257.615) on food intake. Tukey's post hoc test for multiple comparisons showed a significant effect of ghrelin (p=0.018), Anamorelin (p=0.012) and HM01 (p<0.001) compared to vehicle, while pairwise comparisons were used to delineate significant timepoints. (A). Comparison of cumulative food intake 24 hours post-dose shows an overall effect of treatment (p=0.018, df=3, F=3.943), with a significant elevation in food intake 24 hours post-dose for HM01, while a trend is observed for anamorelin (p=0.061) and ghrelin is no longer significant (B). Graphs represents the mean ± SEM. Statistical significance was determined using repeated measures ANOVA for overall effect of treatment, time and time*treatment.Tukey's post-hoc test was used for multiple comparisons, while pairwise comparisons carried out for individual timepoints; statistical significance is depicted as ***p < 0.001, **p < 0.01 and *p < 0.05.*

## 4.11 Saccharin Preference test

Next, naïve rats were individually housed in cages with *ad libitum* access to food and habituated to a 2-bottle choice paradigm with the choice of normal drinking water or drinking water containing 0.1% saccharin. At the onset of the experiment rats were IP injected with either saline, anamorelin or HM01. Overall fluid consumption did not differ between experimental groups (Figure 4.7B). However, there was a significant effect of treatment (p=0.0056, df=2, F=9.727) over a 24 hour period revealed by ANOVA at the same timepoint (pairwise comparisons; Anamorelin p=<0.001, HM01 p=0.035). Furthermore, repeated measures ANOVA showed an overall effect of time (p=<0.001, df=3, F=11.115) and treatment*time (p=0.044, df=6, F=2.366) over the experiment (Fig 4.7A). The preference for saccharin was significantly reduced in rats treated with ghrelin ligands anamorelin and HM01 as shown by Tukey's post-hoc test for multiple comparisons.



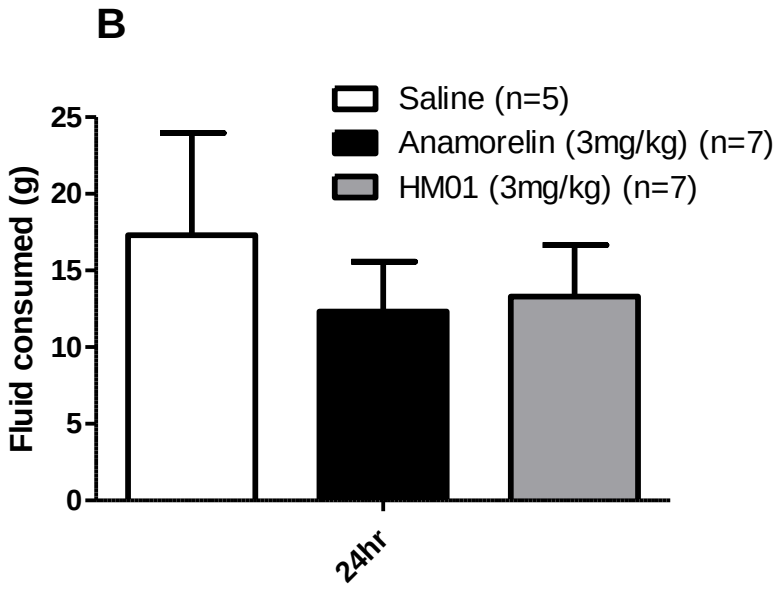
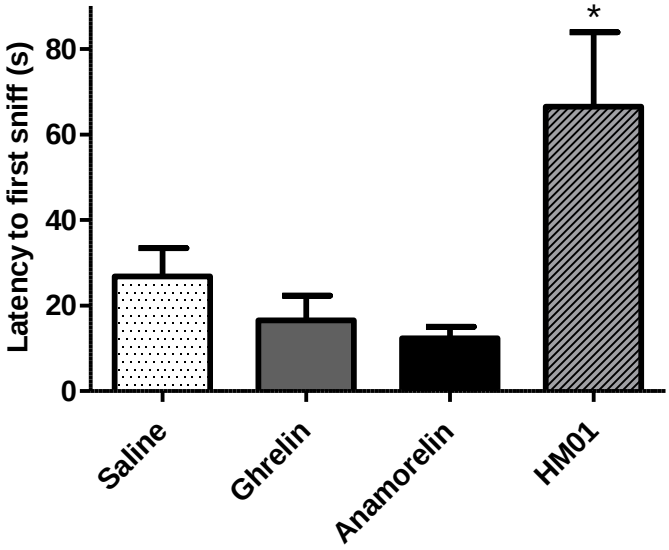



***Figure 4.7. Paradoxical effect of HM01 and Anamorelin on intake of a rewarding, non-caloric saccharin solution***. *Preference for a 0.1% saccharin solution vs. regular water in male sprague-dawley rats was determined following intraperitoneal (IP) injection with 0.3mg/kg body weight of ghrelin, or 3mg/kg of anamorelin or HM01 over a 24 hour period. Saccharin preference was determined at regular intervals after dosing. Overall significant reduction in the preference for saccharin solution was observed for anamorelin vs. saline (p= <0.01) and for HM01 (p= <0.05)(overall significant effect of treatment (p=0.0056, df=2, F=9.727) over a 24 hour period) (A). No overall differences in fluid consumption was observed between treatment groups (B). Graphs represents the mean ± SEM. A repeated measures ANOVA using Tukey's post-hoc test was used to determine overall statistical significance; depicted as **p < 0.01 and *p < 0.05.*

## 4.12 Female Urine Sniffing test

The female urine sniffing test (FUST), typically used to assess anhedonia and depressive-like characteristics in rodents (Malkesman, Scattoni et al. 2010, Burokas, Arboleya et al. 2017), can be used as a surrogate for reward system assessment in a natural, non-invasive approach that is not confounded by differences in metabolic status, calories and/or gustation. HM01 treated mice had a significantly lower number of sniffing interactions with a female urine stimulus than control mice, in addition to displaying an increased latency to sniffing (Figure 4.8). There was no difference in either the number of interactions or the latency to sniffing between anamorelin and control. These results indicate that HM01-treated mice show aversive-like behaviour towards a rewarding stimulus, indicating a paradoxical reward-related deficit induced by the compound. Noteworthy, anamorelin, though displaying the same trend in the saccharin preference test, does not induce the same behavioural phenotype in the FUST.



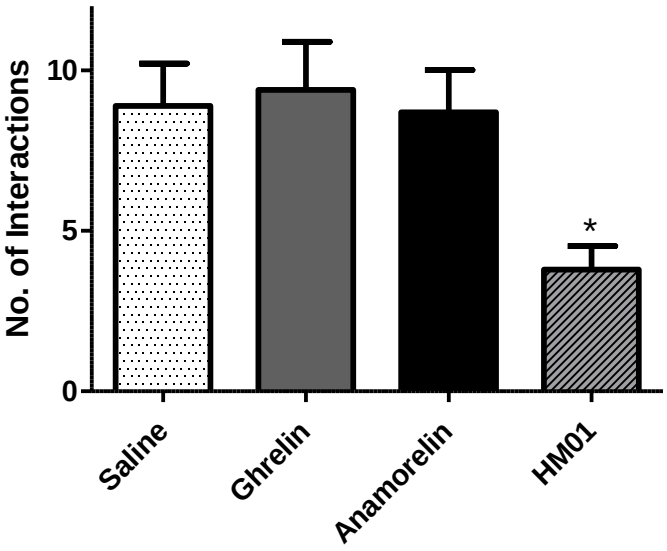



***Figure 4.8. Effect of ghrelin, HM01 and Anamorelin on female urine sniffing test****. Interaction of male C57/Bl6 mice with a rewarding odour (female urine) was determined following intraperitoneal (IP) injection with 0.3mg/kg body weight of ghrelin, or 3mg/kg of anamorelin or HM01. An overall significant increase in the latency to first interaction with female urine reward (A), as well as a decrease in the total number of discrete interactions with the stimulus was observed for HM01(B). A one-way ANOVA using Tukey's post-hoc test was used to determine overall statistical significance; depicted as *p < 0.05.*

# Discussion

Due to its ability to increase food intake and promote adiposity, ghrelin and the GHSR-1a have been pharmacological targets for disorders of appetite such as CACS (DeBoer 2011, von Haehling and Anker 2014). Ghrelin treatment provides a reliable orexigenic and anabolic effect across species, however, since it's discovery by Kojima and colleagues in 1999, only one ghrelin agonist, anamorelin, is close to gaining regulatory approval for CACS (Garcia 2017). Potential barriers to therapeutic success to date have been recently reviewed (Howick, Griffin et al. 2017). One such barrier to the success of ghrelin therapeutics is the ligand-dependent biased signalling exerted by the GHSR-1a, which, upon activation, sends downstream signalling via Gαq- dependent, Gαi/o-dependent or β-arrestin-dependent signalling (Ramirez, Oeffelen et al. , M'Kadmi, Leyris et al. 2015). Moreover, the expression of the GHSR-1a *in vivo* has led to increasing consideration of the biodistribution of GHSR-1a ligands (Howick, Griffin et al. 2017, Mohammadi, Pietra et al. 2018). Here, we investigate anamorelin and HM01, two novel synthetic GHSR-1a ligands. Though these ligands have already shown promising effects on food intake in preclinical and clinical studies, their biased signalling and biodistribution in relation to appetite and reward remains unexplored.

Firstly, GHSR-1a activation by anamorelin and HM01 was investigated in the context of predicting functional outcome based on downstream signalling. Though native ghrelin activates the full complement of signalling, differences in functional selectivity of synthetic ligands toward diverse signalling pathways can have a crucial impact on the ultimate effect observed *in vivo.* Recent evidence has highlighted the behavioural significance of this promiscuous signalling; Gαq-dependent downstream signalling was pinpointed as the major effector in relation to food intake (Mende, Hundahl et al. 2018). Here, the previously unassessed signalling behaviour of anamorelin and HM01 were assessed using calcium mobilization, IP-one, β-arrestin recruitment, and receptor internalization assays. As expected, both ligands produced a strong agonist effect on the calcium mobilization assay compared with ghrelin, in a concentration-dependent manner. The IP-1 assay confirmed the efficacy of the synthetic ligands on the Gαq signalling-pathway as seen with the calcium mobilization

assay. As for the $Ca^{2+}$ mobilization assay, anamorelin and HM01 produced a strong agonist effect compared with ghrelin, in a concentration-dependent manner. Desensitization and internalization of the receptor into endosomal vesicles provides a pivotal feedback loop preventing overstimulation through the GHSR-1a. Predictably, clear GHSR-1a internalization was observed after treatment with ghrelin, anamorelin and HM01. The internalization was concentration dependent with a pattern of potencies aligned with those reported above, but interestingly the $E_{max}$ reached by anamorelin (126%) is much higher than that of ghrelin (74%) and HM01 (69%). This indicates likely GHSR-1a desensitization and amelioration of the *in vivo* effect after treatment with anamorelin, but not HM01. Furthermore, β-arrestin which also functions in the desensitization of GHSR-1a aligns with the previous internalization results as the $E_{max}$ reached by anamorelin (153%) is again much higher than that of ghrelin (107%) and HM01 (89%).

Further to pharmacodynamic differences, biodistribution of ghrelin ligands is an important consideration and is poised to play a key role in future ghrelin research given the widespread GHSR-1a expression in key nodes of the reward system (Edwards and Abizaid 2017, Howick, Griffin et al. 2017). Native ghrelin administration exerts differential effects on neuronal activation depending on whether it is administered peripherally or centrally(Edwards and Abizaid 2017). It follows that HM01, a centrally penetrant compound, would have a differing neuronal activation profile to anamorelin or native ghrelin due to differing distribution *in vivo*. Indeed, it has recently been shown that HM01 has a more potent colokinetic effect compared to a peripherally active GHSR-1 ligand due to its central penetrance (Mohammadi, Pietra et al. 2018). In line with this, significantly elevated c-Fos immunoreactivity, potentially indicative of greater neuronal activation, was noted for HM01 in the LH and the VTA, both of which are key areas in the reward pathway which are not peripherally accessible. Furthermore, the NAccSh showed elevated activity for HM01 only and not ghrelin or anamorelin. c-Fos staining also expectedly showed elevated activation in the Arc for ghrelin, anamorelin and HM01.This c-Fos activation profile indicates a divergent activation of reward-related areas with a brain penetrant ghrelin agonist. This bolsters the theory that central penetrance could lead to greater efficacy of ghrelin therapeutics, through GHSR-1a signalling in reward centres.

Both anamorelin and HM01 show high potency on $G_q$ signalling which is known to be the main signalling pathway responsible for eliciting changes in food intake (Mende, Hundahl et al. 2018). Therefore, *in vivo* effects of the ligands were assessed in an acute food intake paradigm. Dose selection of anamorelin and HM01 was based upon previous publications, while ghrelin was chosen as a positive control based on the seminal paper by Wren et. al (Wren, Small et al. 2001, Pietra, Takeda et al. 2014, Mohammadi, Pietra et al. 2018). The amount of standard rodent chow consumed after administration of a GHSR-1a ligand, or saline vehicle, was monitored over a 24-hour period (Figure 4.6). The orexigenic effect exerted by ghrelin, anamorelin and HM01 are consistent with the c-Fos activation profiles in hypothalamic arc sections. Notably, HM01 elicits a robust elevation in food intake compared to both ghrelin and anamorelin. It is tempting to speculate that the greater efficacy of HM01 in this respect may be due to the BBB penetrability of HM01 compared to the non-penetrant anamorelin. In line with this, HM01 elicits greater activation in the LH and the VTA compared to anamorelin. Moreover, there is no greater magnitude of arcuate neuronal activation by HM01 than anamorelin or ghrelin. Furthermore, there were no appreciable differences between the ligands on GH output (Figure 14, Appendix B) while a higher dose (10mg/kg) of anamorelin and HM01 failed to elicit greater increases in food intake over the 7-hour time frame (data not shown), indicating a plateau in the orexigenic effect of both compounds. One obvious caveat to this speculation is the higher potency of HM01 in *in vitro* assays, therefore further studies must be carried out in order to conclusively prove this theory. Nevertheless, this is the first time that both ligands have been compared head to head over an acute period in a food intake paradigm.

Next, behavioural effects of anamorelin and HM01 were investigated on the reward system using the Female Urine Sniffing Test (FUST) and Saccharin Preference Test (SPT) paradigms. Ghrelin treatment has been shown in the literature to increase the preference for a saccharin solution in rodents (Disse, Bussier et al. 2010). A non-caloric 0.1% saccharin was used as it was a concentration previously shown to cause a robust but not maximal preference in consumption in rats (Sclafani, Bahrani et al. 2010). Overall fluid consumption did not differ between experimental groups, however the preference for saccharin was significantly reduced in rats treated with

ghrelin ligands anamorelin and HM01. Furthermore, the FUST was used to quantify interaction time with a rewarding olfactory stimulus, another behavioural measure of reward system activation (Malkesman, Scattoni et al. 2010). HM01 treated mice had a lower number of sniffing interactions with a female urine stimulus than control mice, in addition to displaying an increased latency to sniffing. These results unexpectedly indicate that HM01-treated mice, but not anamorelin treated, show aversive-like behaviour towards a rewarding stimulus. Therefore, while food intake is robustly increased by both treatments, an unexpected paradoxical reduction in reward-related behaviour was observed. Seemingly, reward paradigms such as SPT and FUST which do not offer caloric benefit in hunger elicit a paradoxical negative response in reward-directed behaviours. This may be indicative of a potential reduction in the palatability of a substance which offers no caloric benefit in times of food seeking, a phenomenon which has been reported (Kawahara, Kawahara et al. 2009). The underlying mechanisms explaining this unexpected behavioural phenomenon require further investigation.

In summary, this paper provides valuable insight into biased signalling and biodistribution of ligands for the GHSR-1a. Accumulating evidence points to the significance of biased signalling in the future development of successful ghrelin-based therapies for appetite modulation (Ramirez, Oeffelen et al. , Mende, Hundahl et al. 2018). Preferentially activating a desired pathway may help to specifically augment desired functional outcomes while limiting side-effects (Bologna, Teoh et al. 2017). Anamorelin and HM01 are potent activators of the $G_q$ pathway and produce a robust effect on food intake *in vivo* via this signalling pathway. HM01 exerts a far greater effect on food intake than anamorelin despite providing no greater hypothalamic activation. We postulated that this may be due to increased brain penetrance of HM01 to the mesolimbic circuitry and the subsequent recruitment of non-homeostatic mechanisms of appetite stimulation. c-Fos immunostaining supports this, with greater activity reported in key input centres of the mesolimbic pathway, such as the LH, VTA and NAccSh after HM01 treatment. However, the behavioural correlates of reward system activation undoubtedly paint a paradoxical picture which needs to be unravelled in further work.

# Conclusion

This paper highlights the potential importance of signalling bias in relation to future ghrelin therapies. HM01 and anamorelin exert potent effects on calcium mobilization, however anamorelin is potentially more susceptible to treatment-induced tolerance than HM01 due to recruitment of β-arrestin and GHSR-1a internalization. Central access of ghrelin ligands, particularly to reward areas of the brain, may be important in eliciting more potent appetite-stimulating effects. c-Fos immunohistochemistry showed greater activation of LH and VTA neurons compared to control for HM01 treated animals only. The greater maximal orexigenic effect of HM01 over anamorelin is potentially due to access of HM01 into the brain penetrance. However, a paradoxical reduction in reward-related behaviour was observed for HM01 in both the SPT and FUST paradigms, while this effect was only evident in the former for anamorelin. This paper provides valuable insight into *in vitro* and *in vivo* aspects of GHSR-1a signalling, however further mechanistic work is needed to conclusively demonstrate the benefit of central penetrance and elucidate paradoxical effects on reward system parameters.

# Chapter 5

# Effects of novel ghrelin ligands, Anamorelin and HM01 on the reward circuitry: A microdialysis study in rodents.

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# Abstract

The endogenous hormone ghrelin is one of the key components in the neuroendocrine system controlling appetite. Receptors for ghrelin (GHSR-1a) are located in the primary site of energy homeostasis, the arcuate nucleus of the hypothalamus, but also in key nodes of the mesolimbic dopaminergic system. Hence, not only does ghrelin act as a barometer for energy balance, but it also functions as a mediator of food reward and the incentive salience applied to food. Manipulation of the ghrelin system can therefore play a pivotal role in altering the top-down regulation of food intake by altering the perception of food palatability. The synthetic ghrelin ligands anamorelin and HM01 have shown promising orexigenic effects in preclinical and clinical studies, however their effect on the reward system has not yet been reported. The aim of the current study was to investigate changes in extracellular DA content in the nucleus accumbens shell (NAccSh) of conscious, freely-feeding Sprague-Dawley rats using a microdialysis paradigm.

Differences in extracellular DA in the NAccSh after treatment with ghrelin, HM01 and anamorelin are reported. Increased NAccSh DA was observed for HM01 compared to control. Therefore, HM01 elicited greater effects on the reward circuitry than anamorelin as measured by DA output in freely-feeding rats. This *in vivo* proof of concept thus highlights the importance of targeting the mesolimbic reward circuitry for enhancing the efficacy of ghrelinergic therapy. The use of brain penetrant ghrelin ligands will prove vital in the future treatment of appetite-related disorders.

# Introduction

Ghrelin is a peripherally-produced endogenous hormone with potent orexigenic and anabolic properties (Kojima, Hosoda et al. 1999, Tschop, Smiley et al. 2000, Inui 2001, Nakazato, Murakami et al. 2001, Müller, Nogueiras et al. 2015, Howick, Griffin et al. 2017). In periods of calorie deprivation, X/A like cells in the stomach release ghrelin into the bloodstream which communicate via neuroendocrine methods with appetite centres in the brain (Kojima, Hosoda et al. 1999). The target receptor for effecting changes in appetite is the growth hormone-secretagogue receptor (GHSR-1a), heavily expressed in the arcuate nucleus of the hypothalamus, the primary fulcrum of energy homeostasis (Inui 2001, Nakazato, Murakami et al. 2001). The mechanism of ghrelin's orexigenic action and it's potential as a target for appetite modulating therapies has been widely described in the literature (Wren, Small et al. 2000, Nakazato, Murakami et al. 2001, Horvath, Castaneda et al. 2003, Naleid, Grace et al. 2005, Müller, Nogueiras et al. 2015, Howick, Griffin et al. 2017).

The decision to eat is largely a conscious process, based on the perception of hunger, the availability of food and the perceived palatability of same (Howick, Griffin et al. 2017). Ghrelin is thought to have a large role in the mesolimbic reward circuitry, thereby modulating the incentive salience of food (Naleid, Grace et al. 2005, Abizaid, Liu et al. 2006). In line with this, the GHSR-1a is expressed in key nodes of the reward system, such as the lateral hypothalamus (LH), ventral tegmental area (VTA) and nucleus accumbens (NAcc) (Zigman, Jones et al. 2006). Abizaid and colleagues demonstrated that ghrelin binds to VTA neurons, triggering dopaminergic neuronal activity, synaptic plasticity and increase turnover of dopamine (Abizaid, Liu et al. 2006). Treatment with ghrelin has been shown to increase the motivation to work for a food reward in rodents (Skibicka, Hansson et al. 2011), as well as shifting the preference from standard chow towards palatable, calorie-dense foods (Egecioglu, Jerlhag et al. 2010). Hence, GHSR-1a signalling in the mesolimbic reward circuitry is considered a major driver in altering perceived palatability of food, and the motivation to obtain it (Egecioglu, Skibicka et al. 2011, Perello and Dickson 2015).

Microdialysis studies have been used previously to investigate ghrelin's role in the reward system by monitoring extracellular dopamine (DA) levels. Dickson's group were the first to show that ghrelin, administered centrally, induced an increase in extracellular DA content in the nucleus accumbens (Jerlhag, Egecioglu et al. 2006). A number of subsequent studies from this group, have also been reported confirming an important role for ghrelin in the reward circuitry (Jerlhag, Egecioglu et al. 2007, Jerlhag 2008, Egecioglu, Jerlhag et al. 2010, Dickson, Egecioglu et al. 2011). However, to the best of our knowledge, no study has been reported investigating the extracellular DA content after treatment with synthetic ghrelin ligands. This is despite the numerous potent synthetic ligands under development as therapeutic agents that have shown promising effects over native ghrelin on food intake and other anabolic parameters (Vodnik, Štrukelj et al. 2016). Despite greater stability and more favourable pharmacokinetics *in vivo*, their reward-related properties have not been investigated in microdialysis studies, however recent work has reported on behavioural changes and central c-Fos immunostaining (Chapter 4).

There has been much debate over the ability of ghrelin to successfully manipulate the reward circuitry despite its lack of BBB penetrability (Cabral, De Francesco et al. 2015, Edwards and Abizaid 2017). Further to this, our group has previously shown a divergent activation of the reward system with ghrelin ligands anamorelin (non-brain penetrant) and HM01 (brain penetrant) (Chapter 4). We hypothesized that this divergent neuronal activation was due to the latter's ability to traverse the BBB and activate GHSR-1a which is present in key nodes of the reward system such as the VTA. Hence, the aim of this study is to establish a microdialysis platform to measure extracellular DA levels in the nucleus accumbens shell, and investigate whether treatment with ghrelin ligands would alter this. The significance of this was intended to give an insight into whether a brain-penetrant synthetic ghrelin agonist would have a greater impact on the "liking" or hedonic aspect of food intake by stimulating greater DA outflow in the NAccSh.

# Materials and Methods

## 5.1 Materials:

High performance liquid chromatography (HPLC) grade acetonitrile, methanol, potassium dihydrogen phosphate and orthophosphoric acid (OPA) were obtained from Fisher Scientific Ireland, Blanchardstown, Dublin, Ireland. Ghrelin (rat) was obtained from Tocris Bioscience, Avonmouth, Bristol, UK (Cat. No. 1465). Anamorelin and HM01 were kindly gifted by Helsinn Therapeutics (Helsinn, Lugano, Switzerland). Guide cannulae and microdialysis probes were purchased from Charles River Den Bosch BV (De Mudden 16, 9747 AW Groningen, Netherlands).

## 5.2 Methods:

### 5.2.1 HPLC Optimization and Validation

The HPLC system consisted of a Shimadzu LC-20AD XR Prominence Pump, CBM-20 communication bus module, SIL-20AC XR Prominence Autosampler, CTO-20A Prominence Column oven (Mason Technology, Cork, Ireland). Shimadzu LC solutions software was coupled to this equipment. The HPLC system was coupled to an electrochemical detector (ED). The detector used was an ESA Coulochem III with a 5041 Amperometric Cell (ESA Analytical, Ltd., Brook Farm, Dorton, Aylesbury, Buckinghamshire, HP18 9NH England). Dialysis samples were injected onto a reversed phase Luna 2.6 µm C18(2) 100 x 5 mm column (Phenomenex), which was protected by Krudkatcher Ultra in-line 0.5µm depth filters (Phenomenex).

### 5.2.2 Analyte identification and quantification

Standard solutions of DA in aCSF were injected onto the HPLC system at different voltages to determine the optimal voltage for detection. Peak height was used as a measure of response and plotted against voltage applied in order to identify the optimal voltage for analyte detection. Calibration curves were constructed to confirm a linear relationship between DA content and peak height in the relevant concentration range. DA was identified by its characteristic retention time (2.5 minutes) as

determined by standard injections which were run at regular intervals during sample analysis. No extraction procedure was necessary for microdialysis samples, so analyte peak height ratios were compared directly with standard injections and expressed as baseline of the individual animal and being expressed as a percentage thereof.

The HPLC-ECD method was adapted from previously described methods (Sato et al., 1994; Frahnert et al., 2003). Before going on the system, the mobile phase was filtered through Millipore 0.22 µm Durapore filters (Millipore, Ireland) and vacuum degassed prior to use. Compounds were eluted isocratically over a 20 min runtime at a flow rate of 0.4 $ml.min^{-1}$ after a 20 µl injection. The column was maintained at a temperature of 26°C and samples/standards were housed at 8°C in the autoinjector prior to analysis. The glassy carbon working electrode combined with a platinum reference electrode (ESA) was operated at a potential of 200mV and a range of 10nA.

### 5.2.3 Stereotaxic guide cannula implantation

Animals were anaesthetized prior to surgery with a ketamine/medetomidine admixture 7.5/5 mg/100g i.p.), with maintenance of anaesthesia achieved by repeating 20-25% of the induction dose at 30-40 minute intervals, as required. Before the surgery took place, depth of breathing as well as the absence of pedal reflex and eye twitch was checked to confirm adequate depth of anaesthesia. Analgesia was provided by peri-operative administration of carprofen (5 $mg.kg^{-1}$ s.c.). Throughout surgical procedures, the body temperature of each rat was maintained using a heating pad. The rats were placed in a stereotaxic frame (Model 900 Small Animal Stereotaxic Instrument, David Kopf Instruments, Bilaney Consultants, St Julians, Sevenoaks, UK) such that the head was flat and centrally aligned. An incision was made from eyes to ears and the skull exposed. Bregma was located and the coordinates for the guide cannula to be implanted were located 1.8mm anterior and 0.8mm lateral to this. A burr hole was made at this location and at another location lateral to this to facilitate the introduction of an anchoring screw. The guide cannule was slowly lowered 5.7 mm from dura into the nucleus accumbens shell (Paxinos and Watson, 1998) and secured with skull screw and dental cement. After this, the surgical site was sutured and a

reversal agent (Atipamezole, 25mg/100g) was administered. Rats were allowed to recover overnight and pain score sheets were maintained as necessary until the microdialysis procedures took place. All experiments were in full accordance with the European Community Council directive (86/609/EEC) and approved by the Animal Experimentation Ethics Committee of University College Cork (AE19130/P062).

### 5.2.4 Microdialysis procedure

On the morning of the microdialysis experiment, the rats were placed in cylindrical plexiglass containers (Instech Laboratories, Plymouth Meeting, PA) filled with bedding. The stylet was gently removed from the guide cannula and the dialysis probe was clicked into place. The inlet tube of the probe was then connected to a fluid swivel (Instech Laboratories, Plymouth Meeting, PA) and artificial cerebrospinal fluid (aCSF: 147 mM NaCl, 1.7 mM $CaCl_2$, 0.9 mM $MgCl_2$, and 4mM KCl) was continuously perfused through each microdialysis probe at a rate of 1.0 $\mu l.min^{-1}$ by a microlitre 'Pico Plus' syringe pump (Harvard Apparatus, Fircroft Way, Edenbridge, Kent, UK). Microdialysis samples were taken for a baseline period of 2 hours before administration of a ghrelin ligand or control. Thereafter, samples were collected at 30 minute intervals for 360 minutes. DA concentrations in the microdialysis samples were determined by HPLC analysis without any extraction procedure, as described above.

### 5.2.5 Probe placement verification

After the microdialysis sampling session was complete, animals were euthanized and brains removed from probe placement verification. Whole brains were gently removed and post-fixed in chilled 4% PFA for 7 days before being transferred to a 30% sucrose solution for 48 hours. After this, brains were immersed in isopentane and snap frozen in liquid nitrogen for storage at -80C until further analysis. Frozen brains were cryo-sectioned on a Leica Cryostat (CM1900) and thaw-mounted on SuperFrost™ microscopic glass slides. Microscope images were taken to confirm that probe placement was correct using the stereotaxic rat atlas for reference (Paxinos, Watson et al. 1980).

### 5.2.6 Data Analysis

Data were analysed and graphs generated using GraphPad Prism software, Microsoft Excel software and IBM SPSS Statistics (v22) software. All means for the standard curve were calculated from the results of at least three independent experiments carried out in triplicate. For the *in vivo* dialysis experiments, data is calculated as a % of baseline readout for each individual subject. Baseline reading was taken as the absolute concentration of the final baseline sample collected immediately prior to the intervention with the ghrelin ligand or control. A repeated-measures ANOVA with Tukey's post hoc test for multiple comparisons was used to determine overall statistical significance of treatment.

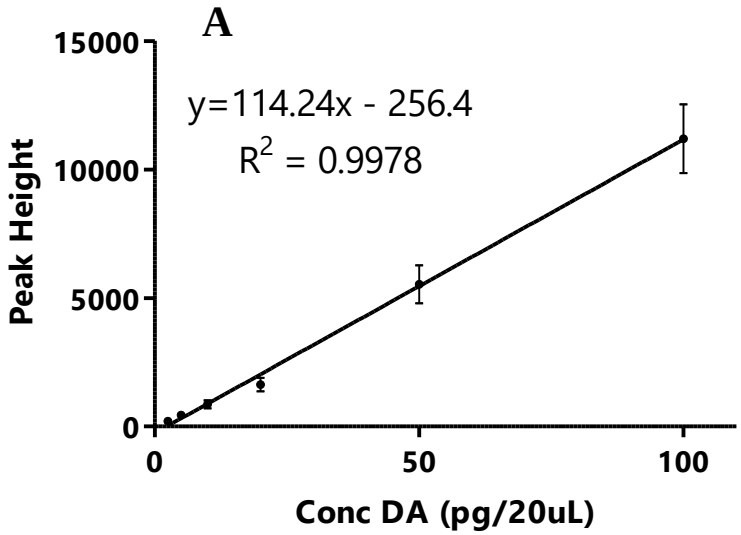
# Results

## 5.3 Effect of ghrelin and ghrelin ligands on dopamine output

Ghrelin has been shown to augment extracellular DA levels in the nucleus accumbens of rodents (Kawahara, Kawahara et al. 2009, Quarta, Di Francesco et al. 2009). Here, the effects of the synthetic ligands anamorelin and HM01 on DA output from the NAccSh of conscious, freely-fed rats were compared to endogenous ghrelin and a saline control.

## 5.4 HPLC Chromatogram

A HPLC based method to quantify DA in aCSF samples was established and validated (Figure 5.1A). A representative chromatogram obtained from the electrochemical detection of a DA standard shows a distinct peak at 2.5 minutes (Figure 5.1B).



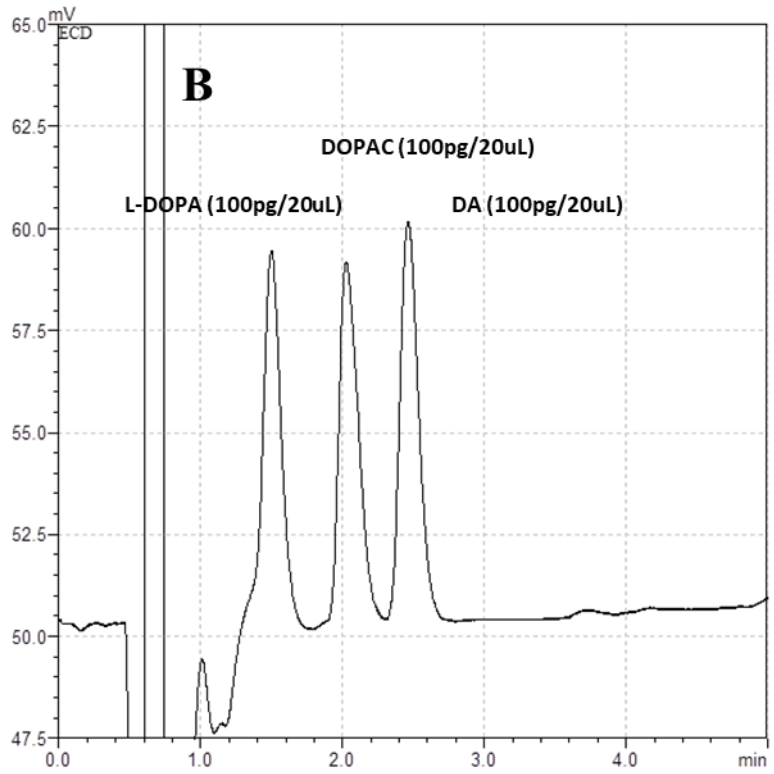



***Figure 5.1. Standard curve of electrochemical detection of dopamine and a representative chromatogram from HPLC analysis:*** *A standard curve of DA concentration, depicted as the magnitude of electrochemical response vs. concentration (Figure 5.1A). A representative chromatogram from an in vitro standard (100pg/20µl) shows a distinct DA peak at 2.5 minutes (Figure 5.1B). Also shown on the chromatogram are the metabolite 3,4 dihydroxyphenylacetic acid (DOPAC) and the precursor levodopa (L-DOPA) in the same concentrations.*

## 5.5 Baseline levels of dopamine

Next, basal levels of DA after treatment with saline vehicle were quantified. As expected, no large peaks or troughs are present although some inherent variability is apparent. Animals were injected after a 2 hour equilibration period and samples were collected every 30 minutes. The absolute baseline concentration was calculated to be 7.08 ± 2.58 pg/20µL per dialysis sample and subsequent readouts are expressed as a percentage thereof (Table 5.1).

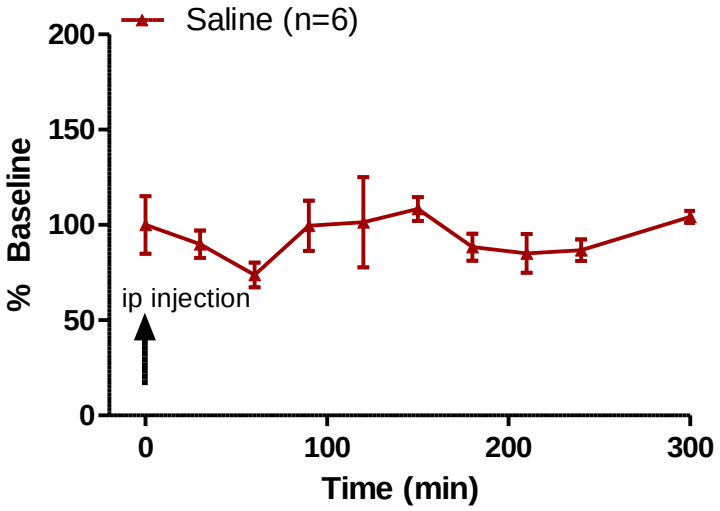



***Figure 5.2. Baseline levels of dopamine after administration of saline ip injection:*** *Change in dopamine levels as a percentage (%) of baseline dopamine content over a 300 minute period of microdialysis. The absolute baseline concentration was calculated to be 7.08 ± 2.58 pg/20µL dialysis sample. Pump flow rate was set at 1.0µl/min. Samples were collected at 30 minute intervals and dopamine concentrations in the microdialysis samples were determined by HPLC analysis.*

## 5.6 Comparing extracellular dopamine levels between treatments

The extent of change in baseline levels of DA was compared between treatments with saline vehicle, ghrelin (0.3mg/kg), HM01 (3mg/kg) or anamorelin (3mg/kg) were quantified. A repeated measures ANOVA revealed an overall significant effect of treatment ($p=0.0115$, $df=3$, $F=4.453$). Tukey's post-hoc test for multiple comparisons showed an overall increase in DA output for HM01 treated animals compared to both saline and anamorelin-treated animals over the 300minute sampling period post-injection. The absolute baseline concentrations of DA are depicted in Table 5.1.

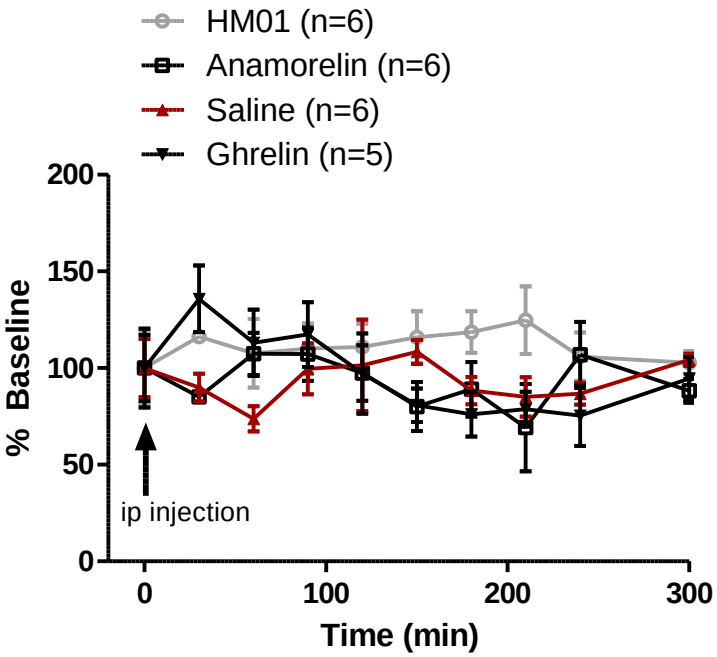



***Figure 5.3. Comparison of change in baseline dopamine levels over a 300 minute period after dosing with saline, ghrelin or ghrelin ligand:*** *Change in dopamine levels as a percentage (%) of baseline dopamine content over a 300 minute period of microdialysis after dosing with either saline, ghrelin, anamorelin or HM01. Flow rate was set at 1.0µl/min. Samples were collected at 30 minute intervals and dopamine concentrations in the microdialysis samples were determined by HPLC analysis.*

***Table 5.1.*** *Average absolute baseline concentration of dopamine (pg/20µL)*

| Treatment | Conc | Std Dev |
|---|---|---|
| Saline | 7.08 | 2.58 |
| Ghrelin* | 6.89 | 2.63 |
| HM01 | 9.08 | 4.53 |
| Anamorelin | 9.7 | 4.47 |

## 5.7Probe placement verification

Microscope images were taken to confirm that probe placement was correct using the stereotaxic rat atlas for reference (Paxinos, Watson et al. 1980). Success rate for correct probe placement was 85%. Probe active membrane is depicted within the dotted line in Figure 5.5.

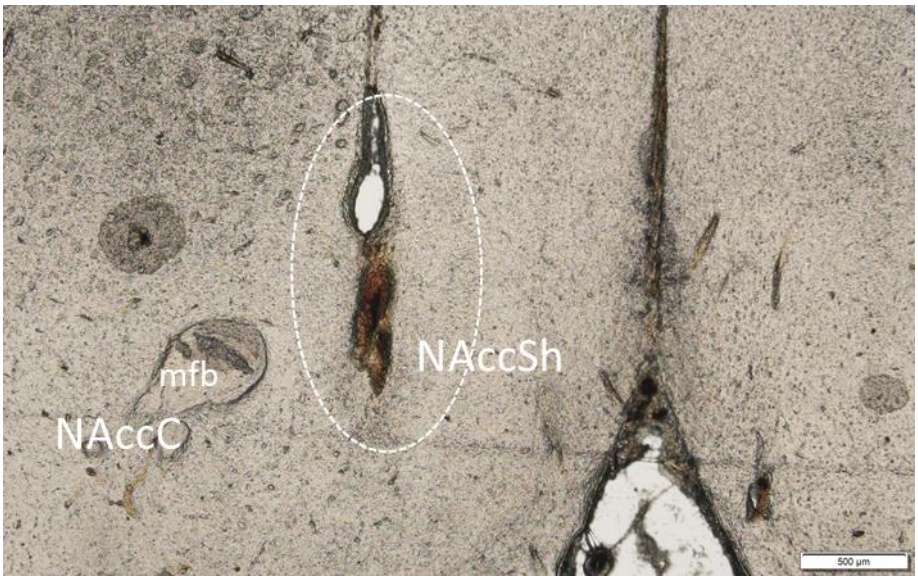



***Figure 5.4. Representative image of probe placement verification*** *Correct probe placement within the NAccSh was confirmed by microscopical analysis using the stereotaxic rat atlas for reference (Paxinos, Watson et al. 1980) (NAccSh = Nucleus accumbens shell, NAccC = Nucleus accumbens core, mfb = medial forebrain bundle).*

# Discussion

Ghrelin remains the only known peripheral hormone with the ability to modulate signalling in the brain in areas associated with food and reward-seeking (Nakazato, Murakami et al. 2001, Andrews 2011, Andrews 2011). Its mechanisms of stimulating homeostatic food intake have been widely reviewed. It has also long been known that "hunger is the best sauce"; a hungry state imparts an increased desire to obtain food (Perello and Dickson 2015). Elevated peripheral ghrelin levels during hunger are experimentally confirmed to increase the perceived palatability and motivation to work for food (Disse, Bussier et al. 2010, Egecioglu, Jerlhag et al. 2010). As such, ghrelin's role in activating the mesolimbic reward circuitry and altering the incentive salience of food has been at the forefront of appetite-modulation research in recent years (Naleid, Grace et al. 2005, Depoortere 2009, Schellekens, Dinan et al. 2013, Perello and Dickson 2015). The mesocorticolimbic DA system is responsible for mediating the rewarding properties of food intake elicited by ghrelin (Naleid, Grace et al. 2005), and a series of publications investigating these effects using microdialysis have been reported (Jerlhag, Egecioglu et al. 2006, Jerlhag, Egecioglu et al. 2007, Egecioglu, Jerlhag et al. 2010, Skibicka, Hansson et al. 2011). Central administration of ghrelin was first shown to modulate *in vivo* DA levels in the NAc (Jerlhag, Egecioglu et al. 2006, Jerlhag, Egecioglu et al. 2007), which was later shown to be dependent on the GHSR-1a in the VTA (Jerlhag, Egecioglu et al. 2009). In addition, a GHSR-1a knockout model showed that DA output in the NAcc, elicited by rewarding food, is GHSR-1a dependent (Egecioglu, Jerlhag et al. 2010).

Numerous synthetic ghrelin ligands have been developed and their ability to increase food intake and GH output has been investigated (Vodnik, Štrukelj et al. 2016). Surprisingly, the effect of these ligands on the reward system has not yet been investigated. Our group previously investigated the impact of ghrelin and synthetic ligands, anamorelin and HM01, on the reward system using behavioural paradigms and c-Fos immunohistochemistry (see Chapter 4). Here, we demonstrated a divergent circuitry at play, which may be dependent on the biodistribution of the ligands. There was a greater ability of HM01 to activate areas of the brain such as the VTA, NAccSh and LH, however paradoxical effects on reward-related behaviour led to the current

investigation of extracellular DA levels in the NAccSh of conscious, freely-feeding rats. In the current microdialysis study, measurements were taken over a total of 6 hours post treatment after a 2 hour baseline equilibration period. HM01 treatment showed an overall significant increase in DA output in the NAccSh compared to both Anamorelin and saline control(Figure 5.3). This is interesting given the hypothesis that a centrally penetrant ghrelin ligand may elicit a greater effect on the reward circuitry than a non-penetrant ghrelin ligand. However, while native ghrelin itself may show a trend towards an effect in the first 120mins of treatment, particularly at the 30minute timepoint, overall there was no effect of ghrelin compared to control. This is despite numerous studies reporting significant effects of ghrelin on DA output from NAcc using microdialysis paradigms, and this warrants further discussion to contextualise our results.

While a significant treatment effect was evident for HM01, there are a number of confounding factors for the current study that merit further discussion. Firstly, in our microdialysis studies, we do not see robust changes in baseline DA levels after ghrelin administration of the magnitude reported in the literature (Jerlhag, Egecioglu et al. 2006, Jerlhag, Egecioglu et al. 2007, Egecioglu, Jerlhag et al. 2010, Skibicka, Hansson et al. 2011). However, the majority of these studies, which have found robust increases after ghrelin treatment are in mice and, notwithstanding the potential species difference, often do not draw a distinction between the core (NAccC) and the shell (NAccSh) of the nucleus accumbens, presumably due to size constraints. Both of these areas are known to serve distinct functions, with the NAccC being responsible for execution of motor function surrounding reward motivation, while the NAccSh dictates the perceived palatability or 'liking' of a reward (Bassareo and Di Chiara 1999, Di Chiara 2002). Therefore, motor components of dopaminergic signalling from the NAccC may underlie the greater increase in DA outflow seen in previous such studies in mice. Indeed, the same studies also report robust increases in locomotor activity (Jerlhag, Egecioglu et al. 2006, Jerlhag, Egecioglu et al. 2007). Conversely, Quarta et. al used a microdialysis paradigm in rats to investigate the differences between the NAccC and NAccSh, reporting that the shell elicited a DA increase after

systemic ghrelin administration while the core had no change in output (Quarta, Di Francesco et al. 2009). Given the species differences and the uncertainty of core contribution, we chose to solely look at the NAccSh in rats due to the widespread reports that ghrelin alters the perceived palatability of food (Egecioglu, Jerlhag et al. 2010, Perello, Sakata et al. 2010, Skibicka, Hansson et al. 2012, Perello and Dickson 2015).

Another potential limitation of the current study may be the procedure taken in establishing baseline conditions. Previous microdialysis setups report an overnight habituation period, however we based our decision to allow a 2-hour equilibration period as per Dickson et. al. (Dickson, Egecioglu et al. 2011) as overnight habituation gave rise to the possibly of probes becoming dislodged from position or becoming blocked. The absolute DA content in the saline-treated group decreased in the 90 minutes post-injection, where no change should have taken place – this indicates an artificially high baseline figure which may be masking subtle changes in DA levels. Furthermore, Kawahara and colleagues used microdialysis to show a bimodal effect of ghrelin on NAccSh depending on whether or not food was present after treatment – food removal after ghrelin was administered induced a decrease in DA output, consistent with an aversive reaction (Kawahara, Kawahara et al. 2009). Conversely, feeding in the post-ghrelin administration induced a robust increase in DA output. Therefore, the variable feeding patterns in the current *ad libitum* feeding experiment may in itself contribute variability to DA response.

Despite the above discussed limitations, the increase in DA output elicited by HM01 is an interesting finding. The fact that HM01 has a greater effect on DA output from the NAccSh compared to anamorelin, ghrelin and saline is consistent with the hypothesis that penetration into the central nervous system allows it to activate GHSR-1a at the level of the reward circuitry, such as in the VTA. This has important consequences for future research in relation to targeting the reward pathway for appetite modulation. Although a trend towards a significant effect of ghrelin in the early stages is evident, overall there is no effect of ghrelin in this paradigm which should be further investigated in future studies.

Overall, the microdialysis results reported here are the first such results for synthetic ghrelin ligands in a conscious, freely-feeding rodent model. Importantly, the aim to establish whether there was a difference in dopaminergic output in this paradigm due to different ghrelin ligand treatment was achieved in the form of greater efficacy of centrally penetrant HM01 vs control. Subsequent studies should leave a longer equilibration period in order to establish a more consistent baseline. The % increases from baseline in this study are likely diluted by the fact that an artificially high baseline is being used, meaning that greater differences are likely to exist than those reported here. Further to this, an examination of higher doses of ligands, in addition to the potential divergent effects of shell vs. core, fasted vs free-access to food, standard chow vs. palatable chow should also be investigated.

# Conclusion

The current study describes a successful microdialysis platform to detect extracellular DA content in the NAccSh of conscious, freely-feeding rats. We report that HM01 stimulates NAccSh DA outflow acutely after administration, while ghrelin and anamorelin fail to elicit such an increase. The fact that the centrally penetrant HM01 elicits a greater DA response over peripherally active anamorelin has important consequences for targeting the reward system for future appetite modulation approaches. However, optimization of the paradigm for establishment of a less variable baseline will help to elucidate these differences in subsequent studies. Furthermore, the standardization of access to food and investigation of rewarding food should be undertaken in the future. Overall, this is an important study which reports novel findings using the technique of microdialyis. These findings can be built upon to further investigate divergent mesolimbic signalling with brain penetrant and non-brain penetrant ghrelin ligands.

# Chapter 6

# General Discussion

# General Discussion

## 6.1Nutraceutical opportunities for early intervention:

Dairy proteins are one of the most abundant sources of bioactive fragments, and there is growing research to indicate that some of these bioactives can have positive effects on appetite and metabolism (Phelan and Kerins 2011, Schellekens, Nongonierma et al. 2014, Torres-Fuentes, Schellekens et al. 2015, Nilaweera, Cabrera-Rubio et al. 2017). However, more translational studies are required to provide insights into the merits and mechanisms of milk-derived bioactives to treat appetite-related disorders. In Chapter 2, we describe for the first time a dairy-derived hydrolysate with inherent capacity to stimulate the GHSR-1a. The casein-derived 1kDa permeate, designated MF1145 (CasHyd for publication) dose-dependently and specifically increased intracellular $Ca^{2+}$ in HEK293A cells heterologously expressing the GHSR-1a (Howick, Wallace-Fitzsimons et al. 2018). Furthermore, we report ghrelin agonistic effects of a whey-based protein derivative, UL 2-141 (FHI-2571 for publication) in Chapter 3 (Howick, Alam et al. 2018). MF1145 displays superior potency (0.27 mg/ml) compared to UL 2-141, however both are considerably less than the endogenous GHSR-1a ligand (0.25 µg/ml), ghrelin (Figure 2.1, Figure 3.1A). This is likely reflective of the fact that the hydrolysates are a mixture of peptides, only some, or one, of which may be active on GHSR-1a.

Furthermore, this *in vitro* activity has been demonstrated to translate to an increase in food intake *in vivo* in a rodent model. Evidence of MF1145 enhancing food intake in healthy male and female SD rats was reported in Chapter 2. Rats orally gavaged with a solution of MF1145 showed significant elevations in food consumption (Figure 2.8). Interestingly, oral delivery had a more robust effect than IP administered MF1145, which failed to show any increase in food intake (Figure 2.7). This may be reflective of the distribution of the GHSR-1a *in vivo*, which is proximal to the intestinal lumen and involved in neuronal signalling to appetite centres in the brain (Howick, Alam et al. 2018). The body of work described in Chapter 2 was the first time a dairy-derived peptide hydrolysate mixture was shown to increase GHSR-1a signalling. Moreover, it was the first time that such an effect was translated *in vivo*

after oral delivery. This is a significant finding given that the field in relation to bioactives and nutraceuticals often relies on *in vitro* bioinformatics, and often a scientific connection is not made between this and clinical use (Howick, Wallace-Fitzsimons et al. 2018).

In reality however, many bioactives are degraded during gastrointestinal transit (de Vos, Faas et al. 2010). Attempts to consolidate the orexigenic effects of MF1145 in Chapter 2 by encapsulation into a gastro-protected delivery vehicle were initially unsuccessful (Figure 2.12). However, the coating platform utilised in this proof-of-concept study was suboptimal, and may have provided a potential barrier to efficacy, as discussed in Chapter 3. As a result, Chapter 3 aimed to provide a robust, sustained-release delivery platform to enable high payload of a bioactive peptide (Howick, Alam et al. 2018). This allowed for further investigation of the orexigenic effects of MF1145, as well as those of whey hydrolysate UL-2-141, in a rodent model. Crucially, the success and scalability of the platform also allowed for the ultimate progression to human studies as part of the Food for Health Ireland work package (Sullivan, Cushen et. al, unpublished).

The above described work is a testament to the potential of exploiting drug delivery technology that is more commonly applied in the pharmaceutical industry, to enhance delivery and bioactivity of nutraceuticals. Conventional drug delivery approaches offer the ability to provide sustained release and gastro-protection of a bioactive peptide which would otherwise be susceptible to acid degradation *in vivo* in the stomach. Although Chapter 3 failed to show increased food intake in rats using the optimised coating strategy (Appendix A), considering the initial promise of Chapter 2 and the inherent limitations of the food intake model discussed (see Limitations section below), the decision was made by Food for Health Ireland to progress this more robust sustained release formulation to human proof-of-concept studies (Sullivan, Cushen et. al, unpublished). Therefore, while work still remains to be revealed in order to elucidate if the orexigenic effect is reproducible and if it is indeed modulated through GHSR-1a signalling, the within described formulation work adds valuable new knowledge to the growing nutraceutical market.

## 6.2Pharmaceutical opportunities for optimization

While there is considerable potential of GHSR-1a modulation, there are still major gaps in our understanding of the mechanisms of action and therapeutic potential of synthetic ghrelin ligands in the clinical treatment of CACS and other disorders of appetite (Howick, Griffin et al. 2017). Though many studies involving treatment with native ghrelin itself have shown promising results (Akamizu, Takaya et al. 2004, Neary, Small et al. 2004, Druce, Wren et al. 2005), synthetic ligands hold the distinct advantage of having longer half-lives and no deactivation through des-acylation (De Ng, Bruera et al. 2016). The pleiotropic pharmacodynamics of the GHSR-1a, as well as heterodimerization and downregulation/internalization of the receptor can ultimately impact on the observed pharmacodynamic effect (M'Kadmi, Leyris et al. 2015, Mende, Hundahl et al. 2018, Ramirez, van Oeffelen et al. 2018). Downstream effects of the GHSR-1a via coupling to different G-proteins have been summarized in Chapter 1 and reviewed in detail elsewhere (Schellekens, Dinan et al. 2013). Notwithstanding this is the widespread tissue distribution of GHSR-1a which lends significance to the biodistribution of ligands *in vivo*.

### 6.2.1 Biased signalling of GHSR-1a

Largely ignored until recently, differences in the functional selectivity of ghrelin ligands can have an impact on the ultimate effect observed *in vivo* (M'Kadmi, Leyris et al. 2015). Selectively activating GHSR-1a signalling with pathway-specific ligands may lead to the development of more successful candidates to treat appetite disorders, while minimising off-target effects. Thus, there is a growing impetus for characterisation of the signalling pathway(s) activated by individual ligands, and their subsequent contribution to the observed behavioural effect. The results of Chapter 4 provide *in vitro* and *in vivo* characterization of two novel, synthetic GHSR-1a agonists. Anamorelin (non-BBB penetrant) and HM01 (BBB penetrant) are potent and selective novel ghrelin receptor agonists with oral bioavailability and longer half-lives than ghrelin (approximately 7 and 4.5 hours respectively). While both compounds have already been reported to have orexigenic and anabolic effects, evidence of their potential biased-signalling was not investigated (Borner, Loi et al. 2016). The

signalling pathways of anamorelin and HM01 were hence characterized and compared to native ghrelin on the GHSR-1a *in vitro,* while effects on appetite and reward-motivated behaviour were also assessed.

Agonist activity of HM01 and anamorelin on the GHSR-1a was shown using both intracellular $Ca^{2+}$ mobilization and IP-one accumulation (Schellekens, van Oeffelen et al. 2013), in HEK293A cells (human embryonic kidney cells) stably expressing the ghrelin receptor tagged with an enhanced green fluorescent protein (GHSR-1a-EGFP). As expected both ligands produced a strong agonist response on both assays. As well as heterogenous signalling and neuroendocrine cross-talk, the expression of the GHSR-1a on the cell membrane is critical to it being a successful therapeutic target (Ramirez, van Oeffelen et al. 2018). However, GPCRs are known to downregulate via receptor internalization or endocytosis causing a subsequent attenuation of effect (Tsao and von Zastrow 2000). Unsurprisingly, the GHSR-1a receptor has been shown to downregulate in response to various stimuli, including ghrelin- and ghrelin-ligand mediated activation (Kaji, Kishimoto et al. 2001, Orkin, New et al. 2003, Camina, Carreira et al. 2004). The effects of anamorelin and HM01 on GHSR-1a internalization into endosomal vehicles were evaluated. Interestingly the $E_{max}$ reached by anamorelin (126%) is much higher than that of ghrelin (74%) and HM01 (69%) as a percentage of control (3.3% FBS). This potentially indicates a tendency towards GHSR-1a desensitization *in vivo* after treatment with anamorelin, but not HM01. These findings are supported by the β-arrestin recruitment assay.

### 6.2.2 Biodistribution and *in vivo* effects of ghrelin ligands

Further to the heterogenous signalling discussed above, the widespread distribution of the GHSR-1a in the various tissues throughout the body is also an important consideration (Figure 1.4). GHSR-1a is present in a multitude of peripheral and central sites; the nature of this widespread distribution being responsible for the plethora of functional outputs (see Figure 1.3). As a result, the administration of ghrelin or a ghrelin ligand will lead to a number of downstream effects spanning the periphery and the central compartment. For appetite modulation therapy, this is a detractive factor resulting in a variety of downstream effects, reducing required

specificity and increasing off-target side-effects (Horvath, Castaneda et al. 2003, Müller, Nogueiras et al. 2015). The non-target tissue effects (e.g. glucose and insulin) (Chabot, Caron et al. 2014) are likely to complicate a delicate homeostatic balance. The fact that both insulin and glucose can have a significant effect on hunger (Woods, Lutz et al. 2006) means that non-specific stimulation of the GHSR-1a in the pancreas likely decreases efficacy of appetite modulation therapy (Lavin, Wittert et al. 1996, Flint, Gregersen et al. 2007). In light of this, it is unsurprising that the biodistribution of ghrelin ligands also would have a significant role to play in determining *in vivo* effects (Howick, Griffin et al. 2017).

In Chapter 4 we investigate the impact of traditional brain penetrability of ghrelin ligands on areas pertaining to appetite and incentive salience. Traditional BBB penetration does not seem to be a key factor for effecting changes to appetite stimulation or growth hormone output due to the endogenous neural machinery to convey elevated peripheral ghrelin levels to higher brain centres from the hypothalamus (Banks 2002, Cabral, Valdivia et al. 2014, Cabral, De Francesco et al. 2015). Indeed, despite being limited to the periphery, anamorelin is under regulatory consideration for the treatment of cancer-anorexia-cachexia syndrome due to its somatotrophic and orexigenic capacity (Garcia 2017). This has also been seen for other non-centrally penetrant compounds (Torsello, Luoni et al. 1998, Laferrere, Abraham et al. 2005). The ability to achieve this functionality despite a lack of central penetrance is the subject of much debate (Cabral, De Francesco et al. 2015, Edwards and Abizaid 2017).

In Chapter 4, robust increases in food intake are reported in rats treated with ghrelin, anamorelin and HM01. HM01 produces a far greater increase in food intake than anamorelin. Interestingly, upon trebling the dose of anamorelin and HM01 in a subsequent food intake study, no greater orexigenic effect is found for either, hence indicating a maximal pharmacodynamic response. GH output, measured as a surrogate for GHSR-1a activation *in vivo*, was found to be equivalent for anamorelin and HM01 groups. Furthermore, c-Fos immunostaining showed no greater activation in the Arc after treatment with HM01 than anamorelin or ghrelin. Therefore, the fact that HM01 is more potent *in vitro* apparently does not account for the greater orexigenic effect

seen *in vivo*. This may be due to the differing biodistribution observed between both compounds. Nevertheless, the acute effects (<24 hours) of both anamorelin and HM01 have not been investigated head to head before, or in comparison with ghrelin. Hence the finding that HM01 exerts a greater effect on food intake than anamorelin is a novel contribution to the field.

It was hypothesised that this may be due to the ability of HM01 to penetrate into the brain and activate the mesolimbic reward pathway. Though the hypothalamus is the traditional site of action for food intake and body weight regulation, the GHSR-1a is also expressed in key nodes of the reward system and contributes to so-called "pleasurable" eating beyond metabolic demand (Abizaid, Liu et al. 2006, Zigman, Jones et al. 2006). Ghrelin's ability to effect this despite a lack of apparent ability to gain access to the brain is an ongoing discussion in the field (Edwards and Abizaid 2017). The biodistribution of the GHSR-1a in areas not immediately accessible to the peripheral circulation has given rise to the theory that central penetrance of ghrelin ligands would be advantageous, by increasing the access of ghrelin ligands to the mesolimbic reward circuitry (Howick, Griffin et al. 2017). No studies have investigated the differential effects of a non-brain-penetrant and a brain-penetrant ghrelin ligand on reward system signalling in this respect. As a result, *ex vivo* immunohistochemistry of reward-related areas was carried out while reward-motivated behavioural assessment and *in vivo* microdialysis were also undertaken.

Immunohistochemistry revealed divergent activation in the reward circuitry for anamorelin and HM01 as measured by c-Fos activation. Significantly elevated c-Fos activation was expectedly noted in the Arc for ghrelin, Anamorelin and HM01, while the NAccSh showed elevated activity for HM01 only, and not ghrelin or anamorelin. Interestingly, only HM01 showed significant elevation in the LH and the VTA, both of which are key areas in the reward pathway which are not peripherally accessible. This c-Fos activation profile indicates a divergent activation of reward-related areas with a brain penetrant ghrelin agonist. This may bolster the theory that central penetrance could lead to greater efficacy of ghrelin therapeutics, through GHSR-1a signalling in reward centres.

Reward-motivated effects were investigated using the Female Urine Sniffing Test (FUST) and Saccharin Preference Test (SPT) paradigms. In the latter the preference for saccharin was significantly reduced in rats treated with ghrelin ligands anamorelin and HM01. Furthermore, the FUST was used to quantify interaction time with a rewarding olfactory stimulus (Malkesman, Scattoni et al. 2010). HM01 treated mice had a lower number of sniffing interactions with a female urine stimulus than control mice, in addition to displaying an increased latency to sniffing. These results indicate that HM01-treated mice, but not anamorelin treated, show aversive-like behaviour towards a rewarding stimulus. Ghrelin treatment has been previously shown in the literature to increase the preference for sweet-taste (Disse, Bussier et al. 2010). Therefore, an unexpected paradoxical reduction in reward-related behaviour was observed. Seemingly, reward paradigms which do not offer caloric benefit in hunger elicit a negative response where a positive one was expected. This may be indicative of a potential reduction in the palatability of a substance which offers no caloric benefit in times of food seeking, a phenomenon which has already been reported albeit in a microdialysis paradigm (Kawahara, Kawahara et al. 2009).

*In vivo* microdialysis investigations were undertaken in Chapter 5 in order to delineate changes in extracellular DA levels after dosing with anamorelin and HM01. The microdialysis results reported here are the first such results for synthetic ghrelin ligands in a conscious, freely-feeding rodent model. The fact that centrally penetrant HM01 has a greater effect on DA output from the NAccSh than anamorelin and ghrelin is consistent with the hypothesis that brain penetrability allows it to activate GHSR-1a at the level of the reward circuitry, such as in the VTA. This work hence highlights important considerations for future research investigating the mesolimbic reward pathway in food intake. The reasons for this may be related to biased agonism, biodistribution or pharmacokinetics and should be considered in future studies.

In summary, ligand-dependant signalling pathways are increasingly recognised for their behavioural significance *in vivo*. In Chapter 4, the *in vitro* signalling pathways activated by both ligands are contrasted and found that anamorelin is potentially more susceptible to treatment-induced tolerance. Furthermore, this is also the first time that work has been done to compare ghrelinergic compounds which

have different abilities to traverse the BBB. The notion that central penetrance may be an important consideration in the biodistribution of ghrelin ligands has gained traction in recent times in the context of reward system activation. Hence, given the divergent activation seen on c-Fos immunoactivation in Chapter 4, it was decided to utilise a microdialysis paradigm in Chapter 5 to further elucidate this hypothesis in a conscious, freely-feeding rodent model. Results prove the theory that central penetrance is an important consideration however further work must be done in order to elucidate the reasons behind this.

## 6.3 Limitations and future perspectives:

### 6.3.1 Nutraceutical approach

Though the results of Chapter 2 and Chapter 3 are both interesting and novel, variability and reproducibility of the studies are particular caveats warranting discussion. These are discussed in further detail in the relevant chapters and are summarized here. In general, food intake studies in rodents are inherently variable and subject to many confounding factors, both internal (baseline satiety levels, stress levels, sleep status) and external (operator skill, local injury from gavage, bulk effect of pellets). Indeed, it is known from in-house experience that the ability of a known orexigen, ghrelin, to stimulate food intake in rodents during the light phase is more consistent the closer to the onset of the light phase, presumably due to the effect of diurnal oscillations (Schellekens et.al, unpublished). All experiments were also carried out in the light phase, when rodents normally would be asleep – circadian fluctuations may serve here as a confounder to assessing true appetite. Oral gavage of a solution of MF1145 demonstrated an increase in food intake over a 7-hour period in healthy male and female SD rats, however this is tempered by a relatively low quantity of food consumed overall compared to other such studies. Inter-experimental variability was evident based on the differences in baseline food consumption between experiments, while attempts to reproduce those seen in rodents proved difficult. The dosing procedure exerted a degree of restraint stress upon the animals, while there is a risk of minor local injury to the oesophagus in gavaged rats which is also likely to impact on food intake.

The bioactive hydrolysate itself is also likely to be highly fragile *in vivo,* due to low gastric pH (discussed in Chapter 2 & 3), as well as intestinal peptidases. Variability in results may well be a consequence of breakdown *in vivo*. Furthermore, the delivery system design incorporated the peptide into a gastro-protected pellet which exerted a degree of processing stress on the peptide, resulting in a loss of ~40% bioactivity. The bulk effect of solid pellets also seems to have imparted a default increase in food intake in both males and females compared to oral solution. While this formulation was useful as proof of concept, process optimisation is required to minimise activity losses, reduce bulk volume and tailor the release profile further *in vivo.*

Bioactives for appetite modulation is a growing field with a high degree of commercial and clinical potential. In particular, there is growing evidence of the role of dairy-derived peptides in this field. Despite the acknowledged limitations in this thesis, hitherto, much of the evidence corroborating the health claims of bioactives and nutraceuticals comes from *in vitro* bioinformatics. Many bioactives are lacking substantial *in vivo* evidence of effect (Li-Chan 2015, Nongonierma and FitzGerald 2015). Placed in this context, this thesis is the first piece of work to report dairy peptide-hydrolysate fractions, MF 1145 and UL-2-141 with intrinsic GHSR-1a agonist activity. Furthermore, it is the first to show promising results of one of the fractions, MF1145, in translating a specific *in vitro* bioactivity with high potency, to a promising biofunctional effect on food intake *in vivo,* suggesting the overall success of this proof of concept work. Future studies should consider the use of automated cages for measuring food consumption as well as the pattern of food intake, locomotor behaviour, urine and faecal pellet output. This would give a broader context to the findings, as well as avoiding experimenter manipulation of cages and allowing for measurement during the dark phase. Furthermore, the use of healthy, normophagic rats should be replaced with elderly rats, or those with mild forms of malnutrition/cachexia in order to mimic the potential clinical scenario.

The formulation paradigm developed in this thesis can also be generalised to other peptides and bioactives in the appetite modulation field, and beyond. This work has developed a simple encapsulation platform capable of delivering solid material

amenable to delivery via oral gavage to rodents. The bioactive material can be encapsulated into a gastro-protected, sustained release vehicle at a high load. A high degree of bioactivity remains after the encapsulation process. Future studies should look at tailoring the release of bioactive to various intestinal areas, and maybe even including permeation enhancers.

### 6.3.2 Pharmaceutical approach

Despite the wealth of evidence discussed above for ghrelin in appetite modulation, the hormone remains as one cog in a complicated appetitive machinery; many complementary and compensatory neuroendocrine responses to changes in GHSR-1a signalling remain as barriers to the overall efficacy of ghrelinergic therapies. After all, despite an abundance of synthetic ghrelin ligands which have been developed over the years, we are faced with a lack of therapeutic success in appetite modulation which is as abundantly clear. Only one such ligand, anamorelin, is close to achieving regulatory approval for treatment of CACS (Garcia 2017). The work of this thesis comes at a time when the field of ghrelin research has realised the significance of GHSR-1a ubiquity and promiscuity, which has only recently come to the fore as barriers to therapeutic success (Chapter 1).

In relation to cumulative food intake studies in Chapter 4, though the same inherent variability applies as described above for Chapter 2 and 3, the robustness of the food intake response elicited by anamorelin and HM01 was such that these limitations are not relevant to our conclusions. Therefore, this section focuses on the caveats associated with investigation of the reward system effects of the ligands. c-Fos immunohistochemistry was carried out and found greater activity of HM01 in key input centres of the mesolimbic pathway, such as the LH and VTA. This indicated a divergent neuronal activation at play between the centrally penetrant HM01 and the non-penetrant anamorelin. However, it must be said that c-Fos is a general marker of neuronal activation and these findings may indicate activation of inhibitory interneurons instead of excitatory neurons. The findings would be better supported by more specific double-staining which would identify the specific sub-type of the neurons activated. Indeed, the activation of inhibitory neurons may explain the

paradoxical findings in the reward-related behaviours. While food intake is robustly increased by both anamorelin and HM01 treatments, an unexpected paradoxical reduction in reward-related behaviour was observed in the FUST and the SPT. This may results from the fact that no calories were present in either of the reward paradigms in question, hence creating an aversive response to the perceived hunger conferred by the agonist treatment similar to a previous microdialysis study (Kawahara, Kawahara et al. 2009). The underlying mechanisms explaining this unexpected behavioural phenomenon require hence require further investigation.

Finally, in Chapter 5 we used microdialysis as a technique to measure extracellular levels of dopamine in the NAccSh. Though this technique found a significant increase in baseline DA elicited by the centrally penetrant HM01, it failed to detect changes in baseline DA levels of the same magnitude reported in the literature (Jerlhag, Egecioglu et al. 2006, Jerlhag, Egecioglu et al. 2007, Egecioglu, Jerlhag et al. 2010, Skibicka, Hansson et al. 2011). The potential reasons for this are discussed in detail in Chapter 5. The current paradigm may need to be optimized slightly to provide for a more consistent baseline before treatment. Furthermore, though microdialysis has been successfully used as a tool in relation to ghrelin in many studies, many of those studies often did not delineate the motor contribution of the NAccC DA. Indeed, though Quarta et. al successfully used a microdialysis paradigm in rats to investigate the differences between the NAccC and NAccSh (Quarta, Di Francesco et al. 2009),

The microdialysis technique quantifies tonic levels of DA over the course of minutes, as opposed to other techniques which monitor phasic changes of DA over the course of seconds. As such, the temporal resolution of DA change may be diluted over the 30 minute sample collection period. Therefore, it could be that a more temporally sensitive fast-scan cyclic voltammetry (FSCV) setup may be more appropriate to quantify DA in this setup. Previous studies have also considered the local tissue damage, potentially disrupting the BBB which may be relevant for central access (de Lange, de Boer et al. 2000). To limit this, we used a guide cannula through which the probe was inserted on the experimental day, however local injury and disruption to the BBB may have occurred. Another important limitation associated with microdialysis

is that absolute levels of DA were not quantified in the extracellular fluid, but rather relative changes in DA compared to baseline were assessed. This became problematic due to our problems in establishing an appropriately stable baseline. Lastly, the decision to eat in humans is largely a top-down decision based on many factors such as social acceptability and emotion among many other factors Therefore, the relevance of extrapolating changes in dialysate DA, as well as behavioural paradigms of reward to humans may be questioned.

The body of work described here adds to the growing field investigating the biased-signalling of the GHSR-1a and its potential impact on pharmacodynamic/behavioural outcomes. Accumulating evidence points to the significance of biased signalling in the future development of successful ghrelin-based therapies for appetite modulation (Ramirez, Oeffelen et al. , Mende, Hundahl et al. 2018). Preferentially activating a desired pathway may help to augment orexigenic capacity while limiting side-effects. Furthermore, given the widespread tissue distribution of GHSR-1a, the biodistribution of ghrelin ligands is another important consideration (Howick, Griffin et al. 2017). Therefore, this work provides a pharmacokinetic slant which is missing from most studies until now. Given the ubiquitous expression of GHSR-1a in the body, the heterogenous downstream signalling, and ability to heterodimerize with other GPCRs, a more holistic approach to targeting the GHSR-1a needs to be adapted. Detailed pharmacokinetic studies for individual ligands would provide a critical tool in order to reconcile with the observed pharmacodynamic effect. Due consideration of the location of activation of GHSR-1a in the body by a ligand, in addition to the downstream pathway activated by that particular ligand should be taken. Relevant parameters which can confound functional output should also be further investigated, such as other appetite-related biomarkers, acyl vs. des-acyl ghrelin levels etc. Lastly, when investigating reward system-specific effects, more of an effort should be made to eliminate the confound of calories that is found in most studies with ghrelin – in hunger, the intake of calories will by default generate a pleasurable neuronal response. In order to get a true estimate of ghrelinergic manipulation on reward system activation further detailed immunohistochemical double-labelling studies should be carried out under different modes of food intake.

## 6.4 Summary and Future perspectives

A PubMed search of the term “ghrelin” reveals close to 10,000 publications. Seemingly, the more research that has taken place, the more questions that have been posed. As such, despite almost two decades and a myriad of research, no GHSR-1a targeting moiety is on the market for a clinical indication. This does not imply a lack of therapeutic potential, but rather serves as a testament to the complexity and heterogeneity of GHSR-1a signalling. The novelty of this thesis is two-fold; firstly, it investigates early intervention for a weakening ghrelin axis using a dietary-derived bioactive. Secondly, it adds to the growing body of evidence which calls for greater understanding of the significance of biased signalling and biodistribution. A new perspective is provided on manipulating top-down control of food intake via centrally penetrant ligands. There is an impetus to build on the work of this thesis and provide more effective appetite modulation therapies in the future.

This thesis has shown for the first time dairy-derived hydrolysate activation on the GHSR-1a and evidence for this effect to translate *in vivo.* Furthermore, the work has added valuable knowledge on drug delivery strategies which could be harnessed for the future investigation of nutraceutical therapies through developing a simple, sustained release coating approach to enable zero-order release of bioactive *in vivo.* Overall this body of work has a high degree of importance to facilitate the development of potential bioactive candidates in the growing field of nutraceutical science. It highlights the importance of active collaboration between food science and pharmaceutical delivery science. Given the demographics of ageing populations and the concomitant rise in comorbid conditions, the attractiveness of a pre-emptive dietary based intervention for early stage cachexia is evident from both a cost and regulatory standpoint.

The thesis also investigates synthetic ghrelin ligands with an emphasis on biodistribution and biased signalling. Biased signalling can have a profound effect on ultimate effect observed *in vivo,* while biodistribution of ligands is coming to the fore in relation to the ubiquity of GHSR-1a tissue distribution. Centrally penetrant ghrelin ligands may thus hold more potential for the treatment of disorders of appetite due to

their ability to attain greater levels in the brain and activate the mesolimbic reward circuitry. In line with this, we report divergent neuronal activation of reward areas after treatment with anamorelin (non-penetrant) and HM01 (penetrant). Furthermore, more robust maximal food intake is reported for HM01 compared to anamorelin. Paradoxical effects on reward system signalling and limitations to our microdialysis platform call for further investigation into the clinical validity of this divergent signalling. Nevertheless, this work provides a valuable contribution to the appetite modulation field and lays a solid foundation for further investigations in the field, particularly in relation of immunohistochemistry and microdialysis.

The work of this thesis advances knowledge in two areas of intervention for disorders of appetite; Chapter 2 and 3 investigate the potential of dairy-derived bioactives for early treatment of cachexia while providing a generalisable platform for bioactive encapsulation in future proof-of-concept studies. Chapters 4 and 5 spotlight biased signalling and biodistribution as important determinants of *in vivo* efficacy. Combined, the work of the thesis provides valuable mechanistic and technical insight to serve as a jump-off point in future investigations in the appetite modulation field.

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# Appendix A

# Supplementary Material to Chapter 3

*Pellet preparation:*

This method has been described in detail in Section 3.4 above.

*Pellet coating:*

This method has been described in detail in Section 3.5 above.

*Cumulative Food intake:*

Male Sprague-Dawley (SD) rats were purchased from Envigo, UK. Rats were 7 to 8 weeks-old when received at the facility. Animals were group-housed (4 rats per cage) in standard holding cages with controlled light-dark cycle (12-h light; lights on at 7:00 a.m.) and in a temperature- (21 ± 1°C) and humidity-controlled (55 ± 10 %) environment. Water and standard lab chow (2018S Teklad Global 18 % Protein Rodent Diet, Envigo, UK) were available *ad libitum*. All experiments were in full accordance with the European Community Council directive (86/609/EEC) and approved by the Animal Experimentation Ethics Committee of University College Cork (B100/3774). Animals were habituated to experimental conditions for a week prior to experiments taking place. On experimental day, animals were administered their respective treatment at the onset of the light phase and then placed in individual cages for duration of food intake monitoring. Food intake was then recorded by weighing the chow at defined intervals. For the gastro-protected pellets, animals were food restricted for a period of 4 hrs before a pre-weighed quantity of chow was added to the cages. The dosing system for pellets consisted of a flexible PVC gavage tube which was filled with a pre-weighed quantity of blank or active pellets. After insertion of the dosing tube a guidewire was used to administer the dose of pellets directly into the stomach.

# Results

*Cumulative Food intake studies:*

The potential for a casein-derived hydrolysate, MF1145, to elicit an orexigenic effect was discussed above in Chapter 2. Furthermore, an optimized formulation strategy which was called for in Chapter 2 was developed in Chapter 3 using UL-2-141 as a model peptide. An aqueous-based bi-layer approach, and also an organic based monolayer approach was developed. Therefore, the orexigenic capabilities of two novel hydrolyates, MF1145 and UL-2-141 were investigated using this optimized formulation. Firstly, the whey-derived hydrolysate was tested in cumulative food intake (CFI) studies. However, no overall differences in food intake were observed. Furthermore, CFI was examined after dosing with encapsulated MF1145, however no differences were noted in total quantity of food consumed here either.

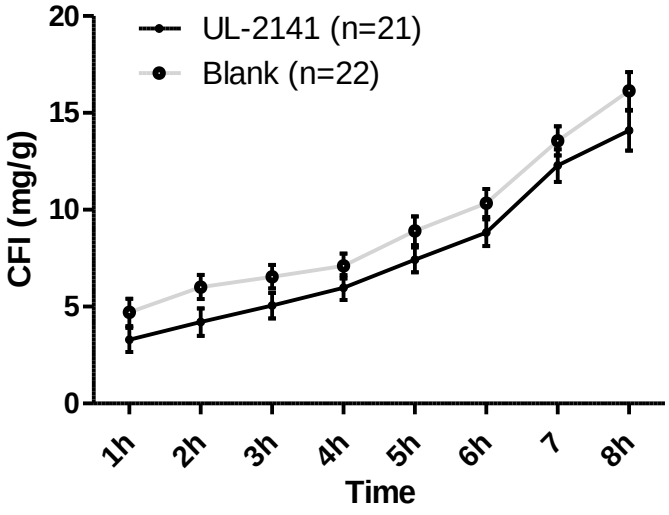


***Figure 1. Cumulative food intake following oral administration of encapsulated dairy derived peptide hydrolysate, UL-2-141.*** *Cumulative food intake (CFI) (regular chow) intake in male sprague-dawley rats was determined following oral administration with 35mg kg$^{-1}$ body weight of encapsulated UL-2-141, over 8 hours. There were no differences noted from control. Data presented as mean ± SEM.*

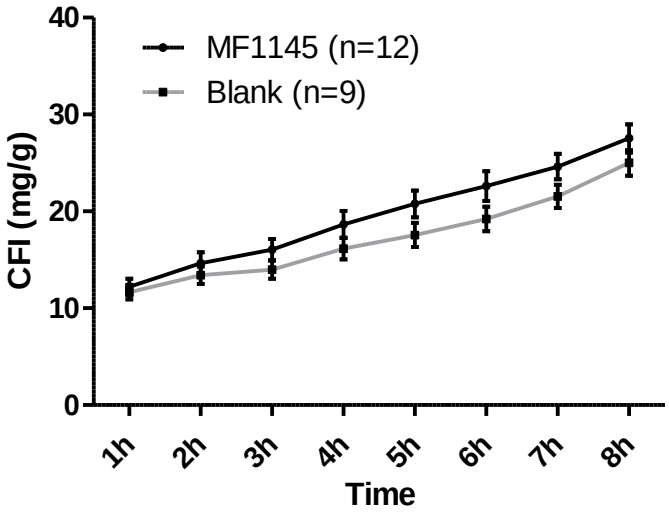


***Figure 2. Cumulative food intake following oral administration of encapsulated dairy derived peptide hydrolysate, MF1145.*** *Cumulative food intake (CFI) (regular chow) intake in male sprague-dawley rats was determined following oral administration with 35mg $kg^{-1}$ body weight of encapsulated MF1145, over 8 hours. There were no differences noted from control. Data presented as mean ± SEM.*

# Discussion

The work from Chapter 2 concluded that there was a potential for appetite modulation, however more reliable sustained release was called for. The area of bioactives for appetite modulation is of growing commercial interest and the potential to address an unmet clinical niche (pre-cachectic states of undernutrition) needs to be backed up with solid scientific evidence. MF1145 (CasHyd, Chapter 2) and UL-2-141 (FHI-2571, Chapter 3) are hydrolysates of casein and whey protein respectively. As discussed in Chapter 2, MF1145 is the 1kDa permeate from a highly reproducible enzymatic hydrolysation process which yields a peptide mixture substantially different from the parent casein. Similarly, UL-2-141 is a peptide hydrolysate which underwent a similar hydrolysation process however the size fraction of the same is larger. MF1145 is more potent than UL-2-141 *in vitro*, likely reflective of the fact that the 1kDa permeate has smaller peptide fractions which correlate with activity on the GHSR-1a.

The ability of bioactive peptides to elicit a beneficial effect *in vivo* is likely to be highly dependent on the use of a gastro-protective delivery system *In vivo* preclinical studies with this bioactive peptide show its potential to act as an appetite stimulant after oral administration. CFI was increased three-fold after 6 hours in male and female SD rats after a single oral dose. In the current study, food intake assessments were repeated using the optimized coating strategy described in Chapter 3. There were no differences noted for UL-2-141 or MF1145 with a sustained-release coating. Importantly, it was shown in Chapter 2 that while activity of MF1145 was eliminated following exposure to gastric pH, administration of MF1145 in a gastro-protected pellet formulation only showed a trend towards increased food intake in both males and females. Therefore, the previous caveats of the paradigm mentioned in Chapter 2 are still due some consideration. Specifically, the potential lack of suitability of the model to assess subtle appetitive changes in a normophagic rat cohort was discussed. Furthermore, the optimized release mechanism of bioactive may be too gradual, and it could be that a burst release similar to endogenous ghrelin is required in order to stimulate a clinically significant orexigenic response. Overall, the potential of dairy bioactives to augment appetite in weakening ghrelin systems is still considered

promising. Future work should examine the orexigenic effect in models of ageing/malnutrition, while also developing release mechanism for burst release in different areas of the intestine.

# Appendix B

# Supplementary material to Chapter 4

# Materials and Methods

*$Ca^{2+}$ mobilization assay*

This method has been described in detail in Section 2.3 above.

*Internalization assay*

This method has been described in detail in Section 4.2.3 above.

*Beta-arrestin recruitment assay*

This method has been described in detail in Section 4.2.4 above.

*Animals*

Male Sprague-Dawley rats (8 weeks) and C57Bl/6 mice (8 weeks) were purchased from Envigo, United Kingdom for use in *in vivo* behavioural experiments. All animals were housed in group cages at 21 ± 1°C, humidity (55± 15 %), outside air ventilation (15±5 cycles/h) with a 12-h light/dark cycle. Animals were acclimatized for at least 1 week before use in experiments. Animals were provided standard chow (Teklad Global 18 % Protein Rodent Diet, Envigo, UK) and tap water *ad libitum*. All experiments were performed in accordance with European guidelines following approval by University College Cork Animal Ethics Experimentation Committee (B100/3774).

*Female Urine Sniffing Test*

The protocol for assessing female urine sniffing behaviour in male C57Bl/6 mice was carried out as per Malkesman et. al (Malkesman, Scattoni et al. 2010). Mice were randomly allocated to one of three treatment groups (Saline, Anamorelin 3mg/kg or HM01 3mg/kg). One week before the test, mice were placed into individual cages in order to remove the effect of single housing on the day of the experiment. On the experimental day, rodents were transferred to a dark room illuminated with a red-light. One hour before the test, mice were habituated to the presence of a cotton-tipped applicator extending into the home cage. 30 mins before the test mice were given an intraperitoneal (IP) injection with the appropriate treatment or saline control. The following protocol took place for each mouse; a three-min exposure to a cotton tip dipped in 60µL sterile water, during which the experimenter left the room and video was recorded for later analysis of duration of interaction, total number of interactions and latency to interact. This was followed by an inter-trial interval of 45 minutes during which no cotton tip was in the cage. Depending on the experimental group, food may or may not have been available *ad libitum* during the intertrial interval. Lastly, a three-minute exposure to a cotton tip dipped in 60µL of urine, freshly collected from a cohort of female mice in estrous, during which the same parameters were recorded.

*Open Field test*

The protocol for the open field test was adapted from a previously described paradigm (Carlini, Monzon et al. 2002). Male SD rats were placed in the centre of a circular arena (90cm diameter) Sixty cm high walls bordered the field. The animals were injected IP 10 mins prior to the behavioural test, after which they were placed into the arena and behaviour continuously monitored for 10 min by a video recorder. The following behavioural components were later measured: locomotion (the total distance travelled), mean velocity, time spent rearing (standing upright on the hind legs), time spent grooming (includes face cleaning, licking, and scratching), and latency to enter the centre zone.

*ELISAs for appetite-related biomarkers*

Next, 2 hours post-dosing with either saline, ghrelin, anamorelin or HM01, rats were euthanized and trunk blood collected. The blood was treated with Pefabloc® 1mg/ml solution in a 1:100 ratio in order to inactivate serum proteases, then the sample was centrifuged at 5000-6000rpm and the plasma removed for snap freezing and storage at -80C. Plasma samples were analysed for Growth Hormone (GH) (Cat. No: EZRMGH-45K) and total Glucagon-Like Peptide (GLP-1) (Cat. No: K150JVC-2), which were purchased from Merck Millipore, Millipore Ireland B.V, Tullagreen, Carrigtwohill, Co. Cork, Ireland and Meso Scale Discovery, 1601 Research Blvd, Rockville, MD 20850 USA, respectively.

# Results

*In vitro assays with HM04 and H1498*

HM04 and H1498 are novel synthetic ligands for the GHSR-1a, the former being a classical competitive antagonist and the latter being an inverse agonist. We tested the ability of both compounds to reduce agonist-mediated calcium mobilization in the same assay. The antagonist behaviour of HM04 on GHSR-1a was shown using intracellular $Ca^{2+}$ mobilization as a measure of downstream GHSR-1a signalling in HEK-293A cells (Schellekens, van Oeffelen et al. 2013), stably expressing the ghrelin receptor tagged with an enhanced green fluorescent protein (GHSR-1a-EGFP). There is a concentration-dependent reduction in the activity response curve for ghrelin ($IC_{50}$ = 2.7 x $10^{-7}$ M), anamorelin ($IC_{50}$ = 8.8 x $10^{-7}$ M), and HM01 ($IC_{50}$ = 7.8 x $10^{-7}$ M) when exposed to HEK-293A cells pre-incubated with increasing concentrations of the antagonist HM04 (Figure 1). Furthermore, no $Ca^{2+}$ influx was observed in wild-type HEK293A cells (HEK293A-WT) cells pre-incubated with HM04 alone. Conversely, there is a concentration dependent increase on the activity response curve for ghrelin, anamorelin and HM01 when exposed to HEK-293A cells pre-incubated with increasing concentrations of the inverse agonist H1498. Therefore, at lower concentrations H1498 antagonizes GHSR-1a activity, however at higher concentrations it has the ability to potentiate the actions of ghrelin, anamorelin and HM01 on the GHSR-1a. This is confirmed by a DRC for ghrelin (Figure 3) whereby the maximal effect of ghrelin is increased two-fold when HEK-293A cells are pre-incubated with a 100nM concentration of H1498 ($E_{max}$ = 200.7%).

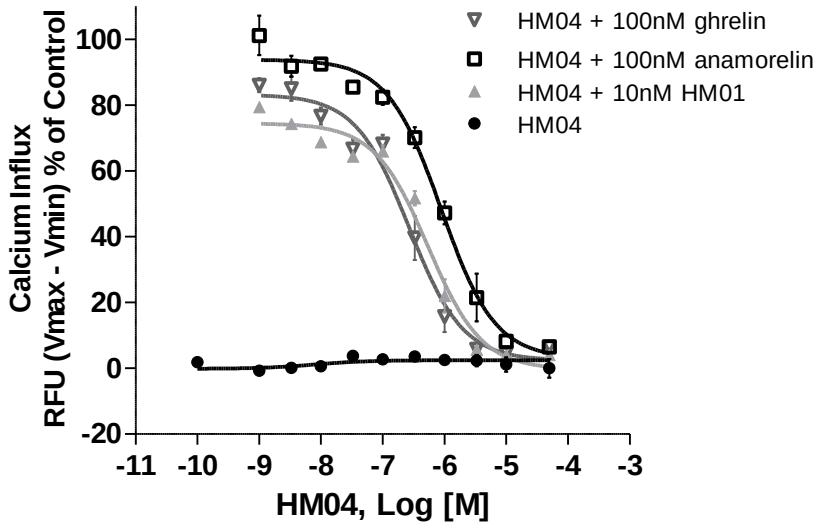


***Figure 1. Concentration-dependent inhibition of GHSR-1a agonist signalling by GHSR-1a antagonist, HM04.*** *Concentration response curves for the endogenous GHSR-1a ligand, ghrelin, and the synthetic GHSR-1a ligands, HM01 and Anamorelin, measured in ghrelin receptor over-expressing HEK293A cells -incubated with increasing concentrations of novel ghrelin receptor antagonist HM04. Intracellular $Ca^{2+}$ increase was depicted as a percentage of maximal $Ca^{2+}$influx in relative fluorescence unit (RFU) as elicited by control (3.3% FBS). Graph represents mean ± SEM of three independent experiments performed in triplicate.*

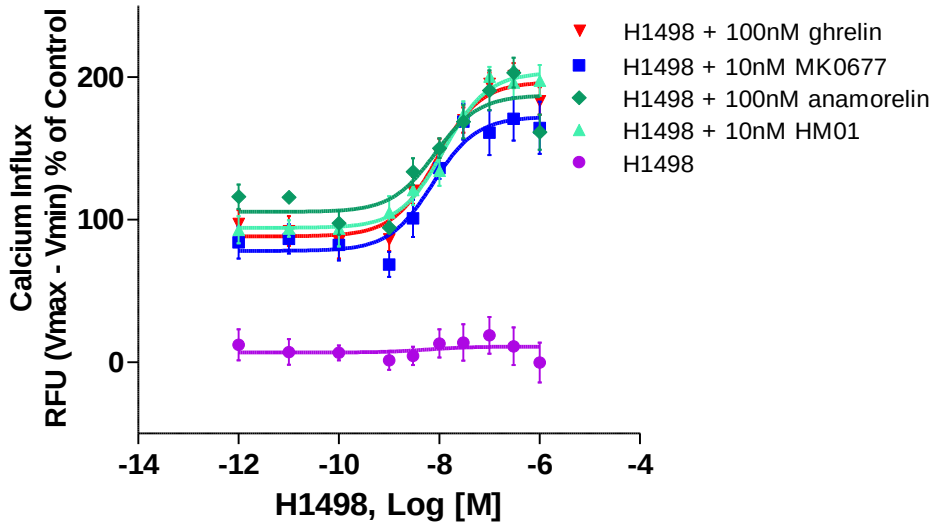


***Figure 2. Concentration-dependent inhibition of GHSR-1a agonist signalling by GHSR-1a inverse agonist, HM1498.*** *Concentration response curves for the endogenous GHSR-1a ligand, ghrelin, and the synthetic GHSR-1a ligands, MK0677, HM01 and Anamorelin, measured in ghrelin receptor over-expressing HEK293A cells -incubated with increasing concentrations of novel ghrelin receptor inverse agonist H1498. Intracellular $Ca^{2+}$ increase was depicted as a percentage of maximal $Ca^{2+}$influx in relative fluorescence unit (RFU) as elicited by control (3.3% FBS). Graph represents mean ± SEM of three independent experiments performed in triplicate.*

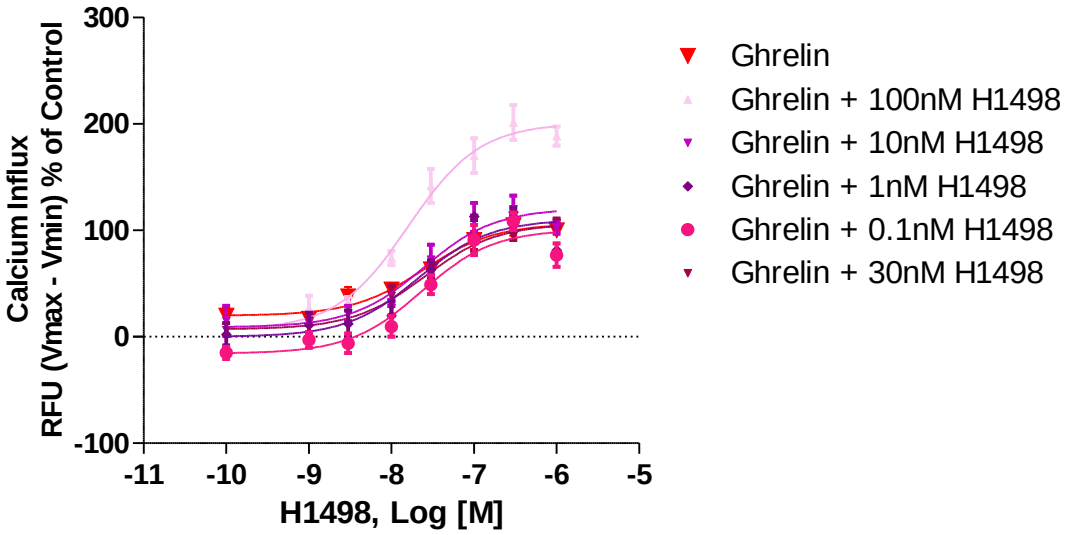


***Figure 3. Concentration-dependent potentiation of ghrelin's action on GHSR-1a mediated calcium mobilization.*** *Concentration response curves for the endogenous GHSR-1a ligand, ghrelinmeasured in ghrelin receptor over-expressing HEK293A cells -incubated with increasing concentrations of novel ghrelin receptor inverse agonist H1498. Intracellular $Ca^{2+}$ increase was depicted as a percentage of maximal $Ca^{2+}$influx in relative fluorescence unit (RFU) as elicited by control (3.3% FBS). Graph represents mean ± SEM of three independent experiments performed in triplicate.*

HM04 was compared with a classical antagonist (JMV 2959) and an inverse agonist (SP) on a receptor internalization assay. Ghrelin (100nM), anamorelin (100nM) and HM01 (10nM) show clear GHSR-1a internalization into endosomal vehicles based on EGFP intensity in the cytoplasm. While JMV (1uM) decreased the receptor internalization slightly, HM04 (1uM) behaved like an inverse agonist and showed evidence of increasing the amount of GHSR-1a expressed at the surface of the cell membrane.

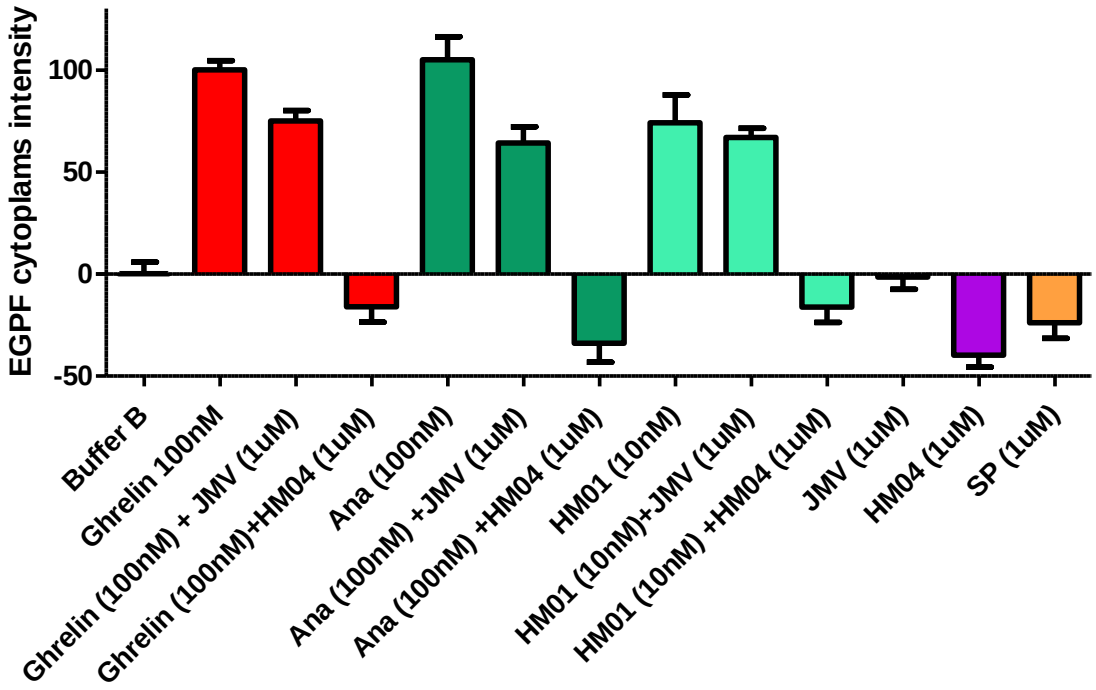


***Figure 4. GHSR-1a internalization assay with JMV-2959, SP and HM04:*** *Internalization of GHSR-1a was quantified after treatment with high dose ghrelin, anamorelin and HM01. The ability of JMV-2959, SP and HM04 to reduce the amount of receptor internalization was quantified. The classical antagonist HM04 was found to act in a similar manner to the inverse agonist, SP, at the concentration tested. There is a shift in the EGFP cytoplasm intensity which is evidence of GHSR-1a recycling to the cell membrane. Intracellular EGFP increase was depicted as a percentage of maximal EGFP intensity as elicited by control (100nM Ghrelin). Graph represents mean ± SEM of four independent experiments performed in triplicate.*

HM04 was compared with JMV in a β-arrestin recruitment assay. Ghrelin (100nM), anamorelin (100nM) and HM01 (10nM) show clear recruitment of the β-arrestin subunit. While no inverse agonist activity is seen for HM04 in this assay, HM04 (1uM) was more potent at halting GHSR-1a mediated β-arrestin recruitment than JMV (1uM).

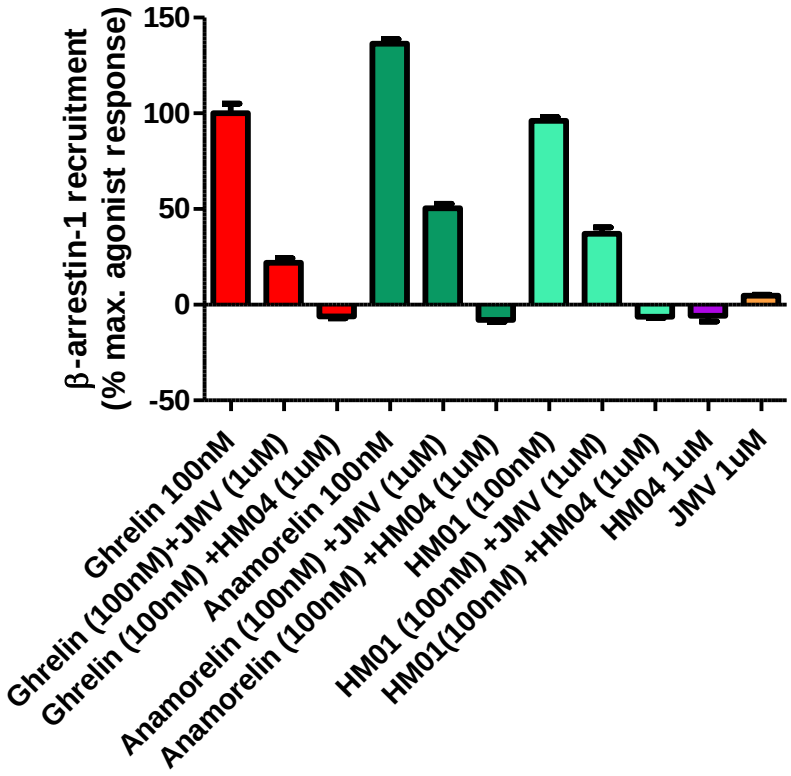


***Figure 5. β-arrestin recruitment assay with JMV-2959, SP and HM04:*** *GHSR-1a mediated β-arrestin recruitment was quantified after treatment with high dose ghrelin, anamorelin and HM01. The ability of JMV-2959 and HM04 to reduce the amount of receptor internalization was quantified based on β-arrestin recruitment. The classical antagonist HM04 was found to act more potently than JMV at reducing β-arrestin recruitment instigated by each agonist. β-arrestin recruitment was depicted as a percentage of maximal EGFP intensity as elicited by control (100nM Ghrelin). Graph represents mean ± SEM of two independent experiments performed in triplicate.*

## Additional behavioural measures

*Female Urine Sniffing test*

Previously we showed a decrease in the number of interactions and the latency to interact with a female urine olfactory stimulus. Given the action of HM01 on food intake, it was postulated that the effect observed may be food-dependent. We hence adapted the FUST paradigm to allow for food to be consumed in the inter-trial interval (food intake data not shown). We report no changes in the behaviour elicited towards the stimulus for control or anamorelin treated animals. However, for HM01 we find a food-dependent effect on the reward-motivated behaviour towards a female urine stimulus. The number of interactions with the stimulus is restored to normal in rats who were allowed eat chow *ad libitum* before the session with the urine stimulus. A trend towards a food-dependent effect is also evident for the latency to interact with the stimulus although n number restrictions makes it difficult to tease out statistical significance.

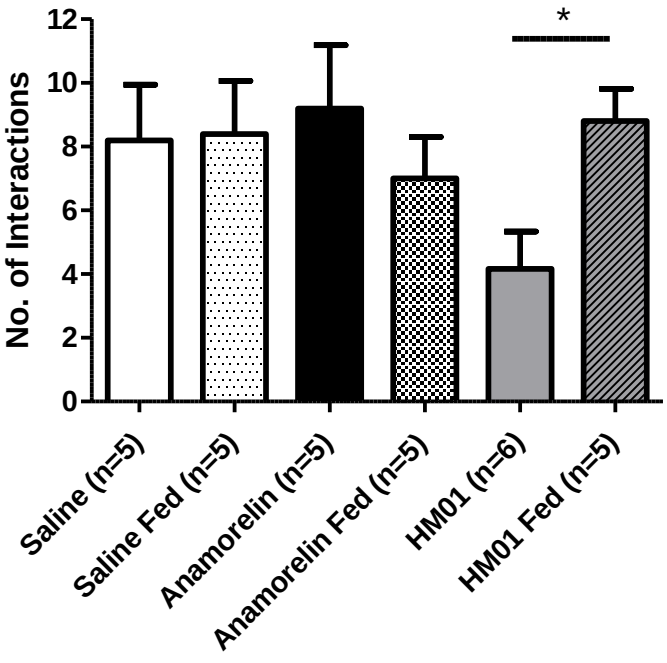

***Figure 6. Effect of ad libitum on effect of HM01-mediated reduction in female urine sniffing behaviour***. *Interaction of male C57/Bl6 mice with a rewarding odour (female urine) was determined following intraperitoneal (IP) injection with saline or 3mg/kg of anamorelin or HM01 and the presentation of food or not in the inter-trial interval. An overall significant decrease in the total time interacting with female urine reward, as well as decreasing the total time interacting with the stimulus was observed for HM01 when food was not present, vs. when animals were able to feed before the urine interaction session. A food-dependent effect of HM01 on reward-related behaviour is proposed.*

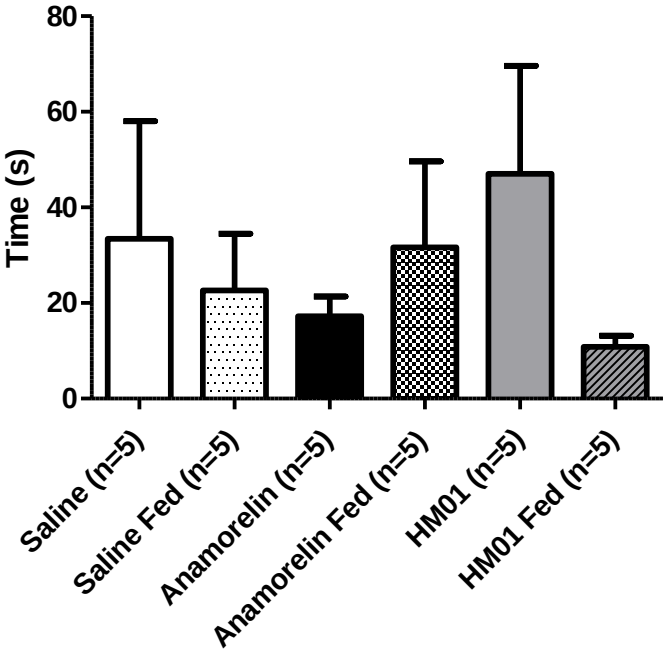


***Figure 7. Effect of ad libitum on effect of HM01-mediated latency to interact with a female urine stimulus*** *Latency of male C57/Bl6 mice to interact with a rewarding odour (female urine) was determined following intraperitoneal (IP) injection with saline or 3mg/kg of anamorelin or HM01 and the presentation of food or not in the inter-trial interval. An overall significant increase in the latency of interaction with a female urine reward was observed for HM01 when food was not present, vs. when animals were able to feed before the urine interaction session. A food-dependent effect of HM01 on reward-related behaviour is proposed.*

The total time spent sniffing (no food present) was also recorded for HM04. This was intended as a control experiment to see if the opposite effect would be seen. There is a trend towards an overall decrease in the interaction time with the urine stimulus in those animals treated with HM04 (Figure 8).

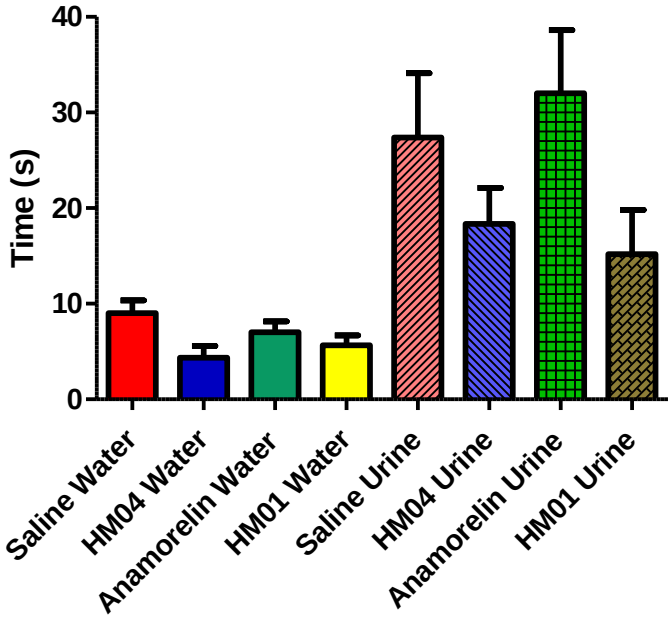


***Figure 8. Total time spent interacting with a female urine stimulus.*** *Interaction of male C57/Bl6 mice with a rewarding odour (female urine) was determined following intraperitoneal (IP) injection with saline or 3mg/kg of anamorelin, HM01 or HM04. A trend towards a decrease in the total time interacting with female urine reward was observed for both HM04 and HM01.*

*Open Field test*

The open field test is a fast and simple test that provides a guide on various behaviours ranging from general locomotor activity to emotional state, such as anxiety of the animal. While it is generally accepted that ghrelin increases locomotor activity and foraging-type behaviour (Jerlhag, Egecioglu et al. 2006, Lockie, McAuley et al. 2017), the question of whether it is anxiolytic or anxiogenic remains under debate (Bali and Jaggi 2016), with both phenomena being reported (Carvajal, Carlini et al. 2009, Jensen, Ratner et al. 2016). As a result, we chose the open-field test paradigm as a quick test to determine if there were any overt differences in locomotor activity, or signs of hyperactivity or lethargy when rats were dosed with ghrelin, anamorelin or HM01.

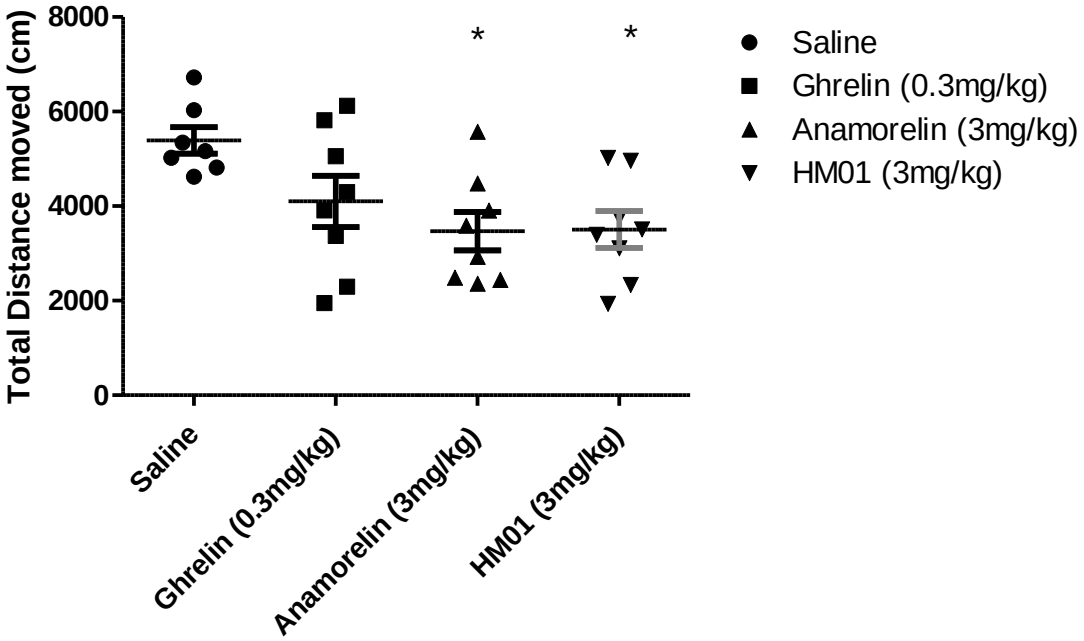


***Figure 9. Total distance moved in open field test****. The total distance moved was recorded automatically using Ethovision software. Anamorelin and HM01 treatment show a significant reduction in total distance moved compared to control. A one-way ANOVA with Tukey's post-hoc test for multiple comparisons was carried out in order to assess statistical significance; (* denotes p = <0.05).*

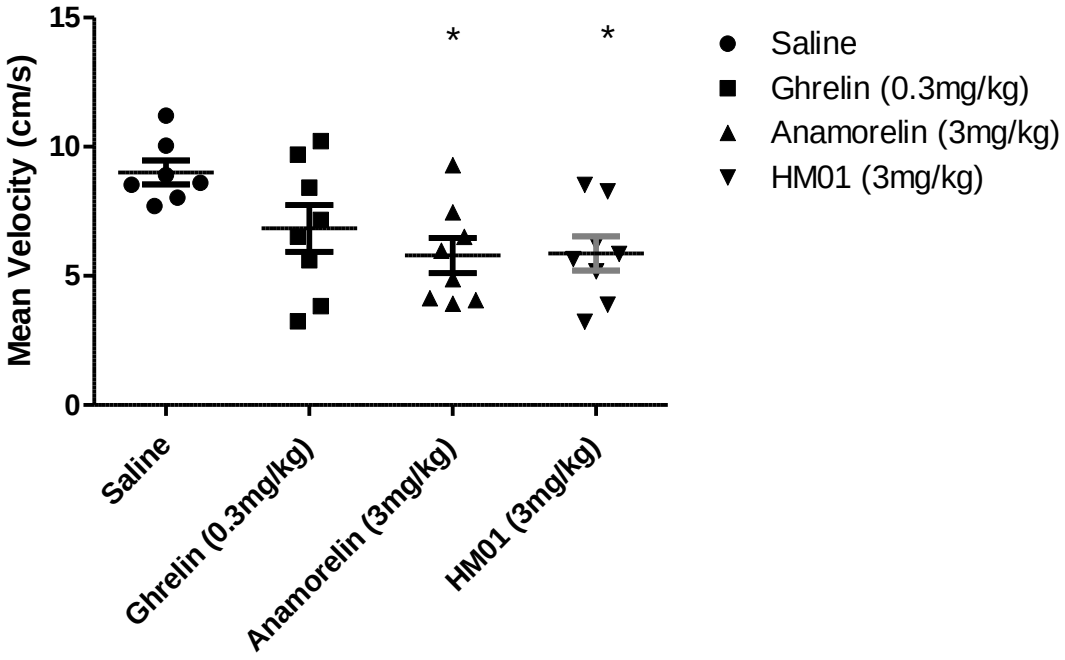


***Figure 10. Mean velocity in open field test****. The mean velocity of movement was recorded automatically using Ethovision software. Anamorelin and HM01 treatment show a significant reduction in mean velocity compared to control. A one-way ANOVA with Tukey's post-hoc test for multiple comparisons was carried out in order to assess statistical significance; (* denotes p = <0.05).*

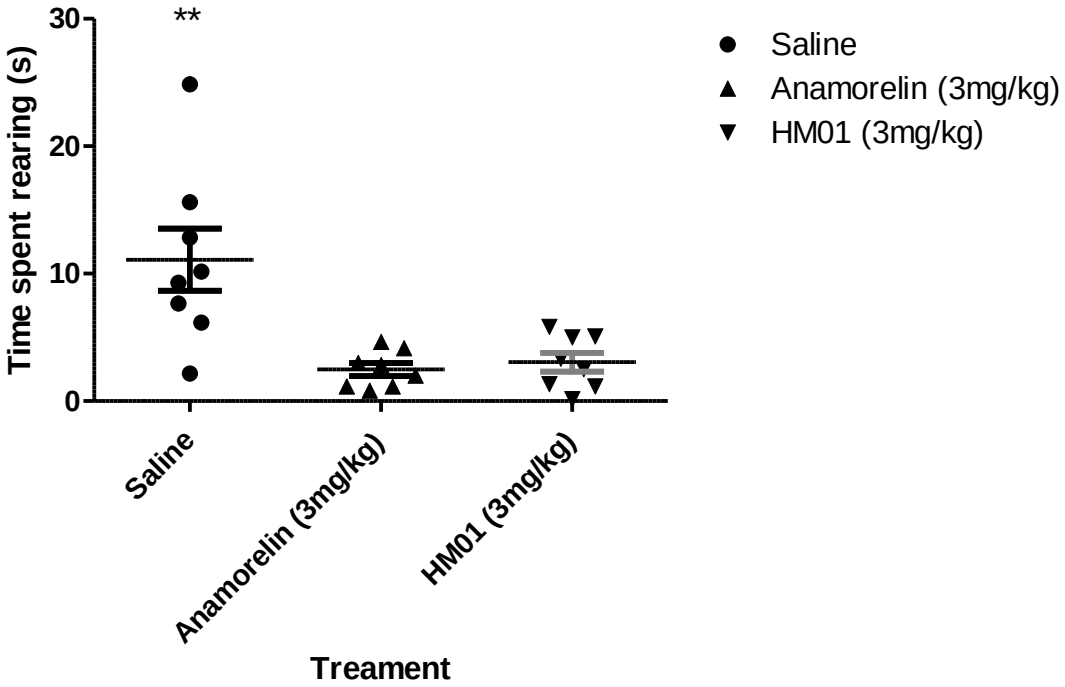

***Figure 11. Time spent rearing***. *The total amount of time spent in a rearing position was assessed and found to be significantly higher for control treated animals compared with anamorelin and HM01. A one-way ANOVA with Tukey's post-hoc test for multiple comparisons was carried out in order to assess statistical significance; (** denotes p = <0.01).*

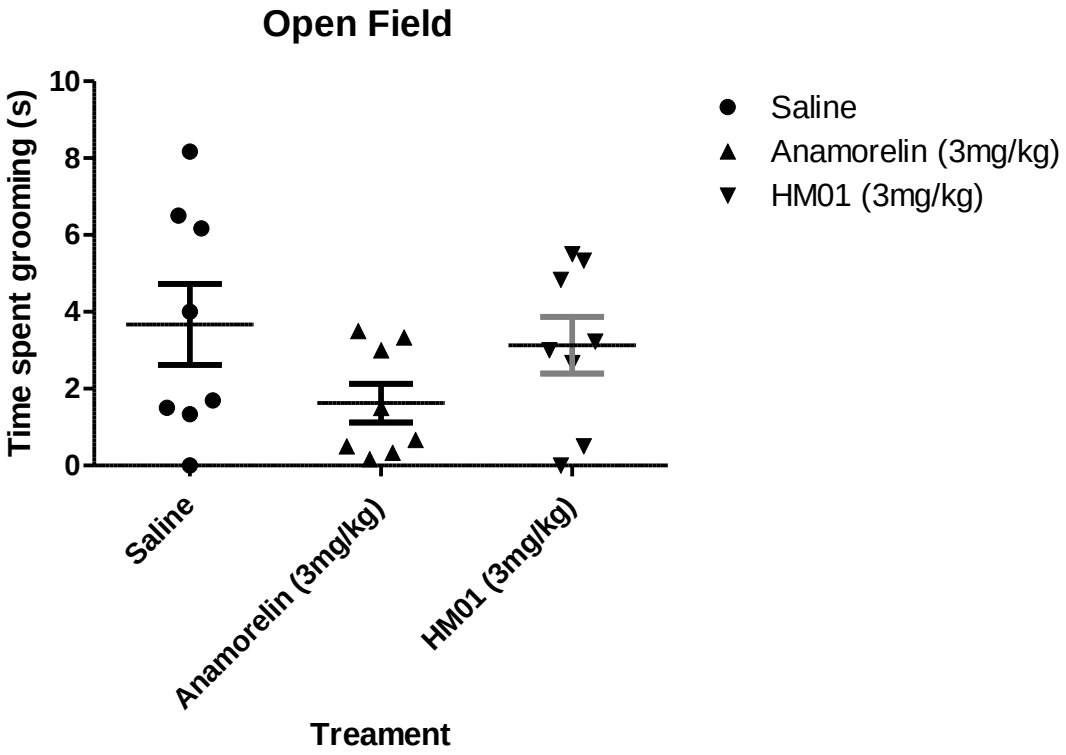


***Figure 12. Time spent grooming***. *There were no significant differences between treatments for the total time spent grooming. A one-way ANOVA with Tukey's post-hoc test for multiple comparisons was carried out in order to assess statistical significance.*

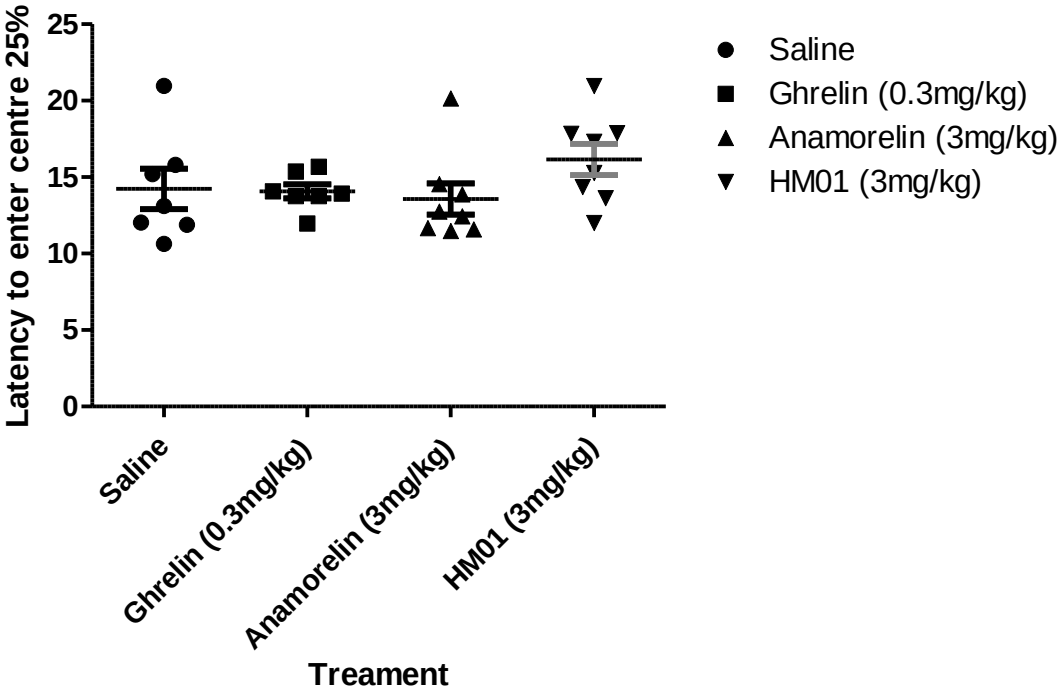


***Figure 13. Latency to enter the centre zone****. The amount of time it took for animals to enter the centre 25% zone of the arena was measured, however no significant differences were noted between treatments. A one-way ANOVA with Tukey's post-hoc test for multiple comparisons was carried out in order to assess statistical significance.*

*Open Field test*

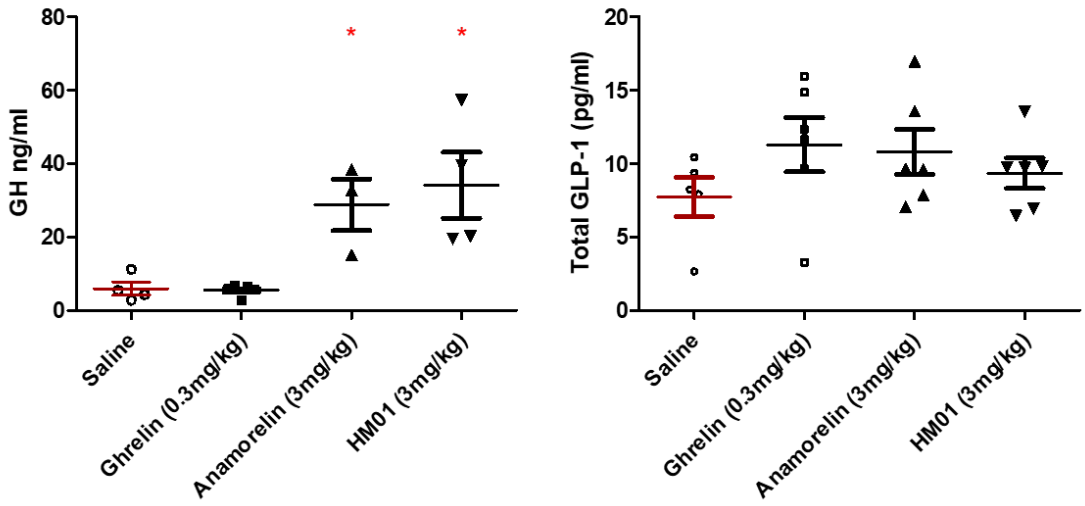


***Figure 14. Effect of ghrelin, HM01 and Anamorelin on growth hormone and total GLP-1 plasma levels.*** *Growth Hormone (GH) and total Glucagon-like Peptide (GLP-1) levels were assessed following intraperitoneal (IP) injection with 0.3mg/kg body weight of ghrelin, or 3mg/kg of anamorelin or HM01. An overall significant increase in GH was observed for anamorelin and HM01 while no increases were observed from ghrelin and saline treatment. Total GLP-1 levels remained unchanged from control. A one-way ANOVA using Tukey's post-hoc test was used to determine overall statistical significance; depicted as *p < 0.05.*

# Discussion

*In vitro*

Both the novel antagonist HM04 and the novel inverse agonist H1498 effectively reduce the activity of ghrelin, anamorelin and HM01 on GHSR-1a mediated $Ca^{2+}$ signalling ($IC_{50}$ range 2.7 – 8.8 $x10^{-7}$ M). Furthermore, the inverse agonist H1498 acts like an antagonist at lower concentrations yet it can potentiate the actions of an agonist at higher concentrations. The maximal effect of ghrelin is increased two-fold ($E_{max}$ = 200.7%) when HEK-293A cells are pre-incubated with a 100nM concentration of H1498 (Figure 3). Interestingly, there is also evidence that HM04 can reverse the internalization of GHSR-1a into endosomal vehicles. An internalization assay showed a reversal of EGFP cytoplasmic intensity, similar to that elicited by the inverse agonist SP, after HEK293A cells were pre-incubated with HM04. The antagonist JMV 2959 did not elicit such an effect (Figure 4). The fact that no inverse agonist activity is seen for HM04 in β-arrestin recruitment may be a limitation of the assay, since it is also not apparent for SP. However, HM04 was effective than JMV (1uM) at reducing GHSR-1a mediated recruitment of the subunit.

*In vivo*

The FUST was used to quantify interaction time with a rewarding olfactory stimulus, another behavioural measure of reward system activation (Malkesman, Scattoni et al. 2010). Before, we showed that HM01 treated mice had a lower number of sniffing interactions with a female urine stimulus than control mice, in addition to displaying an increased latency to sniffing. These results indicate that HM01-treated mice, but not anamorelin treated, show aversive-like behaviour towards a rewarding stimulus. Since HM01 elicited a large orexigenic effect it was postulated that this behaviour may be food-dependent, and that reward paradigms which do not offer the opportunity for caloric intake in hunger elicit a negative response on the reward system (Kawahara, Kawahara et al. 2009). Indeed, this paradoxical effect on reward was highlighted when the antagonist HM04 exerted a similar effect to the agonist HM01 (Figure 8). Accordingly, we modified our experimental design to include a group

which were allowed *ad libitum* food intake in the home cage during the inter-trial interval. Interestingly, the fed group normalized their behaviour towards the rewarding stimulus (Figure 6 & 7). This indicates a food-dependent effect on reward system activation which should be considered in the context of future work on ghrelin and the reward circuitry.

The open field test was also utilized as a general tool to assess for overt locomotor differences induced by anamorelin or HM01 which may impact on food intake. Although ghrelin would be expected to increase locomotion and food-seeking, this was not seen here (Figure 9 & 10). Although a significant reduction in total distance moved and mean velocity was observed for anamorelin and HM01, this was not deemed to be clinically significant and later food intake studies showed a robust effect on food intake corroborated this. Although the open field test can also be used as a barometer for anxiety-like behaviour (Hall 1934), there has been conflicting reports of anxiogenic (Carlini, Monzon et al. 2002, Carvajal, Carlini et al. 2009) and anxiolytic effects (Jensen, Ratner et al. 2016) of ghrelin in this paradigm. We observed no differences in latency to enter the centre zone of the maze indicating no differences in anxiety in this particular paradigm.

# Appendix C

# Additional information on techniques

*Pellet preparation*

- •Requisite quantities of materials were weighed out using scales and weigh boat. Initial process optimization studies trialled a range of different ratios of hydrolysate to microcrystalline cellulose (MCC).
- •Materials were placed in a large zip-loc bag and manually blended for 1 minute.
- •Powder was then added to Kenwood planetary mixing bowl and mixed at minimum setting for a further 5 minutes.
- The resulting blend was granulated by gradual addition of deionized water under constant agitation by planetary mixer at minimum setting. Initial process optimization studies trialled a range of moisture content.
- Deionized water was added via a spray bottle to atomize the water for homogenous dispersion of moisture & to avoid clumping of protein. Water was added every 15 seconds, mixing was stopped periodically every 2-3 minutes to scrape material off the side of the bowl. Weight of water added was monitored.
- End point of granulation was determined visually or when the required percentage water content had been attained.
- Granulate was weighed after water addition was completed and was manually added to the Caleva Extruder 20 (RPM set at 16). Depending on how easy/difficult the mixture is to extrude, addition may be faster/slower. This is always determined visually. Any jams in the extruder were recorded and manually removed.
- Extrudate was funnelled into spheronizer @ 1200-1750 rpm for 1-2 minutes. (rpm and time varied during process optimization).
- Product was allowed to dry @40C/room temp for 24 hours OR in the fluidized bed coater @ room temperature for 15 minutes with maximum airflow.
- Spray coating is the process by which a protective coating is sprayed onto a bed of fluidized pellets. The high airflow and temperature evaporates the solvent to leave a uniform coating on the pellets

*Pellet coating*

- •Pellets to be coated were charged to the coater and the coater reassembled.

- Spray coating was carried out in bottom spray (Wurster) mode, according to the below parameters.

| **Parameter** | **Setting** |
|---|---|
| Nozzle Air | 16.6 psi |
| Airflow | 290-300 L/min |
| Preheating time | 10 minutes |
| Feed rate | 0.25 g/min (variable depending on drying) |
| Inlet temperature | 65-80C |
| Outlet temperature | 50 – 55C |
| Spray pattern | Dependent on coating & drying |

- Duration of spray coating process was determined by the theoretical % of coating required, and the weight of pellets added to the coater. Container of coating solution was under constant agitation @900 – 1000 RPM on magnetic stirrer at room temperature. Container was weighed before and after coating process to determine weight of solution sprayed on the pellets.

* During the coating process coater was observed for correct flow of pellets, and to make sure there were no jams in the atomizer. After pellets were removed, solution was sprayed through the coater to make sure atomizer was still patent (i.e. unblocked) and the system was left flush through with warm water for 20-30 minutes to avoid solids precipitating in the solution feed line.

*In vivo cumulative food intake experiments*

- Randomization into relevant groups was carried out.
- Rats were weighed day prior to (and on the day of) experiment. Dose of active to be received was calculated (35 – 50 mg/kg). Weight of pellets needed to be gavaged was then calculated based on weight.
- Tubes (13ga X 90mm, Instech Laboratories) were pre-filled with pellets and refrigerated the day before the experiment. Each tube was labelled by rat number. Briefly, balance was tared with a plastic gavage tube sealed at one end with parafilm. Pellets were manually loaded to required weight. Other end of the tube was then sealed with parafilm/tin foil. Care was taken when loading tubes to omit pellets which were irregular or likely in any way to obstruct the flow of pellets out of the tube. A number of spare tubes for each group were prepared in case of blockage/ animal biting tube.
- Animals were gavaged with pellets on the morning of experiment and placed in individual cages for duration of food intake monitoring. Cages were randomized appropriately on rack.
- All parafilm was removed from gavage tube and a small amount of Vaseline was added to the end of the tube to prevent pellets from falling out and to ease insertion of tube. A 1ml syringe barrel (without plunger) was attached to tube and cut to length such that the metal guidewire terminates at the end of the dosing tube when inserted (Important to not cause injury to the animal).
- Each animal was restrained, and dosing tube inserted. The guidewire was then fed down through the syringe barrel and dosing tube to push pellets out of tube into the stomach.
- Animals were food restricted for a period of up to 4 hrs before a pre-weighed quantity of chow was added to the cages. The food pellets were placed at the back-left corner of the cage for each animal.
- Food intake was then recorded by weighing the chow at defined intervals.
- New food was weighed and given if levels were running low, or if pellets became wet due to urination.
- After experiment was completed animals were rehoused in home cages.

*Stereotaxic surgery*

- Anaesthetize rat using ketamine/medetomidine admixture and set up on stereotaxic frame.
- Make one incision from eyes to ears using scalpel.
- Use 4 clips to pull back skin from four corners.
- Use cotton buds to move away layer above skull.
- Dip cotton buds in adrenaline/hydrogen peroxide and dab over skull to constrict vessels and stop bleeding. This should allow time for bregma to appear.
- Mark bregma with marker under magnifying glass.
- Take AP (anterior posterior) and ML (medial-lateral) co-ordinates for bregma.
- Take AP (anterior posterior) and ML (medial-lateral) co-ordinates for lambda.
- Check that DV co-ordinates for bregma and lambda are +/- 0.3mm.
- Readjust nose bar to level the skull if there is a discrepancy greater than this.
- Calculate required co-ordinates (Nucleus accumbens shell) based on co-ordinates in rat brain atlas (AP +1.7, ML +/- 0.8, DV 7.2 + probe glue spot length (from dura).
- Move needle on stereotaxic frame to that point.
- Mark skull with needle.
- Use marker to mark spot also if required.
- Mark one other spot to the back of the area for screws which lend support .
- Drill all three holes (drill speed 4, only until drill pops back up). Make sure the microdialysis probe hole for entry is completely free of membrane by putting a needle in past skull to pop any remaining membrane.
- Place screws in spare hole, holding with forceps and screwing with screwdriver (may require downward pressure).
- Replace needle with guide cannula. Ensure it is pointed straight down, with adequate clearance from the positioning block to allow space for gluing.
- Insert guide cannula, making sure it enters center of the hole and is pointed straight downward.
- As it is being lowered in, stop just when it is in past the level of the skull, and take DV (dorsal ventral) co-ordinates. Calculate how far into the brain the cannula should be placed and gradually lower it in.
- Mix some cement and solvent in a petri dish and start to cement the cannula in place, using the screws for support. This will require many layers of glue.
- Once cement is dry, stitch up head.

- Give appropriate dose of reversal agent (atipamezole) and subcutaneous carprofen for post-operative analgesia.

**Surgery tools:**

Stereotaxic frame, arm and ear bars
Anaesthetics + doses
Scalpel
Bulldog Clips
Cotton buds
Blue needle
Probe
Cannula
Screws
Forceps
Screwdriver
Drill
Marker
Dental cement
Petri dish
Spatula
Shaver
Suture kit (including spring scissors)
Optic cable light
Heating pad
Orange Needle for IP anaesthesia and SC analgesia

**Sampling:**

Microdialysis cage apparatus
Allen keys
Bedding, Chow, Water bottle
Tape
HPLC glass vial inserts
aCSF (0.2uM filtered)
Spring for over probes
Glue mix
Tubing (PE50/PE25, PE10)
Harvard Syringe micropump
Blu-Tack
Balance for food
Drug aliquots
Ethanol (Probe activation)

**Additional:**

Experimental sheet & card
Pain sheets/Surgical sheets
Ketamine/ Atipamezole/Medetomidine
Carprofen
Saline 0.9%/EtOh

Adrenaline 0.1%/ Hydrogen Peroxide